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DRUG INTERACTIONS WITH THE NUCLEOSIDE TRANSPORT SYSTEM IN ERYTHROCYTES AND CNS MEMBRANES

by

ⓒ JAMES RICHARD HAMMOND

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TO MY PARENTS

AND MY WIFE CATHERINE

ABSTRACT

The nucleoside transport system plays a key role in the cellular action of many physiological and synthetic nucleosides, and inhibition of nucleoside transport has been invoked in postulates concerning the mechanism of action of several drugs.

Characteristics of the nucleoside transport system of erythrocytes and CNS membranes have been explored in the present study through an examination of the site-specific binding of a potent and selective inhibitor of nucleoside transport, nitrobenzylthioinosine (NBMPR). This high affinity probe has been shown to bind to transport inhibitory sites of functional nucleoside transporters in erythrocyte membranes, and inhibition of nucleoside transport was proportional to site occupancy.

In fresh human erythrocytes, and those of rabbit, mouse, rat, and guinea pig, NBMPR bound with high affinity to a single class of sites. The maximal binding capacity, which was species dependent, was proportional to the maximal velocity of zero-*trans* influx of uridine.

Inhibition constants for several agents, derived from competition experiments with NBMPR, were similar to those obtained from the direct measurement of the inhibition of uridine transport. Therefore, inhibition of the binding of NBMPR by a test compound is predictive of the ability of that compound to inhibit nucleoside transport.

As measurement of nucleoside transport rates in CNS structures presents several methodological difficulties, the utility of NBMPR as a probe for the CNS nucleoside transport system was investigated.

NBMPR bound with high affinity to sites in guinea pig CNS membranes. Regional differences in site density were apparent, but subcellular distribution of the sites was relatively uniform. Characteristics of these sites, including: (1) the affinities of recognized and putative nucleoside transport inhibitors, such as 6-thiopurine nucleosides, coronary vasodilators, benzodiazepines, phenothiazines, and β-carbolines, (2) rates of association and drug-induced changes in rates of dissociation of NBMPR, and (3) inhibitor- and permeant-induced enthalpy and entropy changes of the binding site complex, were similar to the characteristics of the NBMPR binding sites located on transport inhibitory elements of the nucleoside transport system in erythrocytes. The order of affinity of agents for NBMPR sites indicated that these sites are unrelated to adenosine receptors and high affinity 'neuronal' and 'non-neuronal' benzodiazepine recognition sites.

Membrane - located high affinity sites for NBMPR were also detected in rat, dog, rabbit, and mouse brain. Kinetic and mass law analysis, and competition experiments with dipyridamole, revealed multiple forms of high affinity sites for NBMPR in several species. A class of site was identified in rat, rabbit, and dog brain which displayed a low affinity for dipyridamole.

These results suggest that NBMPR is a useful probe for the nucleoside transport system of erythrocytes and CNS membranes. Multiple forms of NBMPR sites may indicate the existence of multiple nucleoside transport systems.

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LIST OF ABBREVIATIONS

NBMPR	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosyl purine (nitrobenzylthioinosine)
Nitrobenzylthioinosine phosphate	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosyl purine 5'-monophosphate (nitrobenzylthioinosine 5'-monophosphate)
Nitrobenzylthioguanosine	2-Amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine
Hydroxynitrobenzylthioguanosine	2-Amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine
ATP	Adenosine 5'-triphosphoric acid
GTP	Guanosine 5'-triphosphoric acid
Cyclic AMP	Adenosine 3',5'-cyclic monophosphate
Ro 5-4864	7-Chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one
Ro 15-1788	Ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5-a] [1,4] benzodiazepine-3-carboxylate

Ro 11-6896(+)	(S)-5-(o-Fluorophenyl)-1,3-dihydro-1,3-dimethyl-7-nitro-2H-1,4-benzodiazepin-2-one
Ro 11-6893(-)	(R)-5-(o-Fluorophenyl)-1,3-dihydro-1,3-dimethyl-7-nitro-2H-1,4-benzodiazepin-2-one
βCCE	Ethyl β-carboline-3-carboxylate
CNS	Central nervous system

I

INTRODUCTION

I. INTRODUCTION

A. GENERAL

Adenosine, a physiological nucleoside, has been attributed a wide variety of regulatory functions both in peripheral and central neuroeffector systems (see reviews by Stone, 1981, and Daly, 1982). These functions include autoregulation of organ blood flow, presynaptic neuromodulation of transmitter release, regulation of cardiac muscle excitability, control of neuronal excitability and regulation of post-synaptic receptor sensitivity. Adenosine appears to produce most of its effects through specific extracellular adenosine receptors (see review by Stone, 1982) and it is removed from the extracellular media by transport into cells followed by enzymatic metabolism (see review by Fox & Kelly, 1978). Inhibition of the nucleoside transport system would, therefore, be expected to potentiate the biological effects of adenosine. Many structurally unrelated, pharmacologically active compounds share the ability to inhibit cellular accumulation of adenosine and potentiate adenosine effects. These compounds include 6-thiopurine nucleosides, coronary vasodilators and benzodiazepines. Inhibitors of the nucleoside transport system would also prevent the cytotoxic effects of various nucleoside analogues which require transport to gain access to their intracellular sites of action.

This introduction commences with a brief review of the biological effects of adenosine and the role of adenosine receptors. This will be followed by an overview of the enzymatic metabolism of adenosine and a detailed description of the nucleoside transport system. An examination of the pharmacology of some recognized and putative nucleoside transport inhibitors will then be undertaken. The final section of this introduction will deal with the problems associated with conventional nucleoside transport studies. In order to circumvent these problems, nitrobenzylthioinosine (NBMPR), a nucleoside transport inhibitor, has been advocated as a high-affinity probe for the study of the nucleoside transport system. Evidence in support of this use of NBMPR will be reviewed.

B. Biological Effects of Adenosine

One of the first regulatory functions of adenosine to be recognized, and the one which has been the most extensively studied, is the metabolic regulation of coronary blood flow (see reviews by Mustafa, 1980, and Berne, 1980). Adenosine is released under conditions of hypoxia and produces a relaxation of vascular smooth muscle (Su, 1975; Schrader & Bardenheuer, 1981). This results in vasodilation and, therefore, increased blood flow to the hypoxic region. Recent reports suggest that prostaglandins may also be involved in the autoregulation of coronary blood flow. Some of these effects of prostaglandins may be mediated through an adenosine mechanism. For instance, the vasoconstricting effect of the prostaglandin $PGF_{2\alpha}$ is inhibited by adenosine (Karmazyn & Dhalla, 1982). Also, prostacyclin (PGI_2), which is known to produce vasodilation (Moncada, 1982), causes the release of adenosine from cardiac tissue (Blass *et al.*, 1980).

Adenosine-mediated control of blood flow is not only confined to the coronary vasculature. It may play a similar role in the regulation of blood flow in renal tissue (Spielman & Thompson, 1982), adipose tissue (Sollevi & Fredholm, 1981), skeletal muscle (Proctor & Duling, 1982) and in the brain (Winn *et al.*, 1981a; Berne *et al.*, 1981).

Release of adenosine from brain tissue has been demonstrated during periods of ischemia (Winn *et al.*, 1979), seizures (Winn *et al.*, 1980), or hypoxia (Winn *et al.*, 1981b), conditions in which increased cerebral blood flow would be beneficial. A smooth muscle relaxant effect of adenosine has also been demonstrated in non-vascular smooth muscle, including that of ileum (Davies *et al.*, 1982) and trachea (Colman, 1976).

Adenosine has a potent depressant action on cardiac function. Negative chronotropic and inotropic effects of adenosine have been reported (Evans *et al.*, 1982), as has an adenosine-induced depression of atrial and ventricular automaticity (Szentmiklosi *et al.*, 1980) and atrioventricular conduction (Belardinelli *et al.*, 1980).

Adenosine also depresses the release of neurotransmitters from both cholinergic and adrenergic nerves (see reviews by Stone, 1981, and Fredholm & Hedqvist, 1980). This presynaptic action of adenosine was first described by Ginsborg and Hirst (1972) who showed that adenosine was able to depress the spontaneous and nerve-evoked release of acetylcholine at the rat phrenic nerve-diaphragm junction. Inhibition of neurotransmitter release by adenosine and related compounds has since been described in a variety of peripheral tissues (Sawynok & Jhamandas, 1976; Clanachan *et al.*, 1977; Clanachan, 1979; Fredholm & Hedqvist, 1980; Stone, 1981; Gustafsson *et al.*, 1981; Ekas *et al.*, 1981; Hom & Lokhandwala, 1981; Wakade & Wakade, 1982) and in the CNS (*vide infra*).

It is will known that ATP is released along with catecholamines upon sympathetic nerve stimulation and it has been suggested that ATP and/or its metabolites, such as adenosine, may function as co-neurotransmitters (Burnstock, 1976; Fredholm & Hedqvist, 1980) or modulators of adrenergic neurotransmission (Fredholm & Hedqvist, 1980; Katsuragi & Su, 1982a). ATP itself may function as a neurotransmitter in nonadrenergic-noncholinergic nerves ('purinergic nerves'?)(Burnstock, 1975; Burnstock, 1979a), although the evidence for this is as yet inconclusive (see reviews by Fresholm & Hedqvist, 1980, and Stone, 1981).

Adenosine may not only influence the release of neurotransmitters but may also regulate the sensitivity of postsynaptic receptors (see review by Fredholm & Hedqvist, 1980). Holck and Marks (1978) suggested that the release of purines from nerve terminals might be important for the maintenance of postsynaptic alpha-adrenoceptor sensitivity. Similarly, histamine receptor sensitivity may also be regulated by adenosine. 4-Methylhistamine, which normally acts selectively on histamine H_2 receptors, also acts on histamine H_1 receptors in the presence of adenosine (Daly, 1976).

Substantial evidence has been compiled in support of the hypothesis that adenosine also functions as a inhibitory neuromodulator in the CNS (see review by Phillis & Wu, 1981c). As in peripheral neuroeffector systems, adenosine appears to inhibit the release of neurotransmitters in the CNS (Harms *et al.*, 1979; Michaelis *et al.*, 1979). Adenosine and related adenine nucleotides have been shown to be potent depressants of neuronal firing in, for example, rat cerebral cortex (Phillis *et al.*, 1979b), guinea pig olfactory cortex (Okada & Kuroda, 1980) and rat hippocampus (Siggins & Schubert, 1981; Lee & Schubert, 1982).

Adenosine and adenosine analogues produce a variety of behavioural effects in laboratory animals (Phillis & Wu, 1981c; Yarbrough &

McGuffin-Clineschmidt, 1981). Sedation is the most common observation (Crawley *et al.*, 1981b; Dunwiddie & Worth, 1982). An anticonvulsant effect of adenosine has also been described (Maitre *et al.*, 1974) and various adenosine analogues antagonize seizures produced by several agents in mice and rats (Dunwiddie & Worth, 1982). These behavioural effects of adenosine are compatible with the inhibitory neuromodulatory role of adenosine described above. Additional evidence for a behaviour modifying role of endogenous adenosine comes from interactions between adenosine and various behaviour modifying drugs such as opiates, benzodiazepines, antidepressants and methylxanthines (see review by Phillis & Wu, 1981c). The behavioural stimulant effects of methylxanthines have been attributed to a blockade of adenosine-induced neuronal depression in the CNS (see reviews by Snyder *et al.*, 1981, and Daly *et al.*, 1981) and high concentrations of some methylxanthines can produce convulsions (Ritchie, 1975). Opiates appear to enhance the release of adenosine from neurones and it has been proposed that adenosine may mediate the neuronal depressant effects of opiate drugs (Perkins & Stone, 1980b; Wu *et al.*, 1982). Potentiation of the extracellular effects of adenosine due to an inhibition of adenosine uptake may be a factor in the therapeutic actions of antidepressants and benzodiazepines (see review by Phillis & Wu, 1982).

C. Adenosine Receptors

Many of the actions of adenosine appear to be mediated through specific extracellular receptors (see review by Stone, 1982b). These receptors have affinity for adenosine and various adenosine analogues and for xanthines such as theophylline (see review by Daly, 1982). Using radiolabelled adenosine, or one of several radiolabelled adenosine analogues, specific receptors for these ligands have been characterized in membranes prepared from dog ventricles (Dutta & Mustafa, 1979), dog coronary arteries (Dutta & Mustafa, 1980), hog carotid arteries (Schutz & Brugger, 1982), rabbit aorta (Ghai & Mustafa, 1982), rat adipose tissue (Malbon *et al.*, 1978), capillaries and pia-arachnoid of rat cerebral cortex (Palmer & Ghai, 1982), and membranes prepared from guinea pig, rat, ox, and bovine brain (Schwabe *et al.*, 1979; Bruns *et al.*, 1980; Williams & Risley, 1980; Newman *et al.*, 1981). The distribution of adenosine receptors in rat brain has been visualized autoradiographically using N^6-cyclohexyl[^{3}H]adenosine (Lewis *et al.*, 1981). Stimulation of adenosine receptors, in general, seems to result in a reduction in intracellular calcium ion availability, possibly mediated through a modulation of cyclic-AMP levels within the cell (see review by Stone, 1982b, and references therein). Activation of adenosine receptors in CNS neurones appears to result ultimately in a hyperpolarization of the cell membrane (Michaelis & Michaelis, 1981).

Adenosine and various adenosine analogues have been shown to increase both cyclic AMP (Sattin & Rall, 1970; Davies *et al.*, 1980b; Nimit *et al.*, 1981) and cyclic GMP (Saito, 1977) levels in brain slices.

Other cells also appear to have adenosine receptors which mediate cyclic AMP accumulation. These include cardiac cells (Huang & Drummond, 1976), platelets (Mills & Smith, 1971) and various cultured cells (Blume *et al.*, 1973; Clark *et al.*, 1974). Regulation of intracellular cyclic AMP levels may be a means through which adenosine produces many of its effects. For instance, adenosine may regulate fat cell metabolism by reducing cyclic AMP accumulation, thereby inhibiting lipolysis (Schwabe *et al.*, 1975; Hjemdahl & Fredholm, 1976)

These stimulatory and inhibitory effects of adenosine on cyclic AMP levels described above indicate that adenosine may produce its effects through at least two distinct mechanisms. In this regard, two distinct types of extracellular adenosine receptors have been characterized. These receptors have been designated as A_1 and A_2 receptors by Van Calker *et al.*,(1979), or as R_i and R_a receptors by Londos *et al.*,(1980). A_1 (R_i) receptors have a high affinity for adenosine and have been shown to mediate an inhibition of adenylate cyclase, whereas A_2 (R_a) receptors have a relatively lower affinity for adenosine and seem to stimulate adenylate cyclase. These two receptor types exhibit a different order of affinity for various adenosine analogues (Londos *et al.*, 1980, and see review by Daly, 1982), and attempts have been made to classify other adenosine receptors as A_1 (R_i) or A_2 (R_a) according to their order of affinity for these various compounds. For example, the adenosine receptors mediating presynaptic inhibition of neurotransmission have been reported to be of the A_1 variety (Paton, 1981) and A_2 receptors, mediating neuronal depression, have been characterized on cerebral cortical neurones

(Newman *et al.*, 1981; Phillis, 1982).

An intracellular adenosine receptor, termed the P-site, has also been described (see review by Daly, 1982). The interaction of adenosine or some adenosine analogues with this P-site appears to result in an inhibition of adenylate cyclase (see review by Daly, 1982).

Xanthines, such as theophylline, antagonize the effects of adenosine which are produced through extracellular adenosine receptors. Theophylline, however, does not block the actions of adenosine on the intracellular P-site (see review by Daly, 1982). The presynaptic inhibitory effects of adenosine on acetylcholine and noradrenaline release can be antagonized by theophylline (Sawynok & Jhamandas, 1976; Clanachan *et al.*, 1977; Fredholm & Hedqvist, 1980; Gustafsson *et al.*, 1981). Adenosine-induced depression of neuronal firing in the CNS can also be blocked by theophylline (Phillis *et al.*, 1979a; Perkins & Stone, 1980a). Furthermore, theophylline and other xanthines can themselves lower the firing threshold of hippocampal neurones (Dunwiddie *et al.*, 1981) presumably by virtue of their ability to block extracellular adenosine receptors (Dunwiddie, 1980; Dunwiddie *et al.*, 1981). This further supports the proposal that endogenous adenosine may play a role in the regulation of neuronal excitability (*vide supra*).

D. Metabolism of Adenosine

Since adenosine appears to produce many of its physiological regulatory effects at extracellular loci, factors which modulate extracellular adenosine concentrations would be expected to modulate these effects. The enzymatic transformations of adenosine occur intracellularly, therefore, inhibition of the nucleoside transport system involved in the cellular accumulation of adenosine may have a major effect on extracellular adenosine concentrations. However, inhibition of intracellular adenosine breakdown or potentiation of adenosine synthesis would, in theory, also lead to an increase in extracellular adenosine concentration due to adenosine efflux through the nucleoside transport system.

One possible pathway for the production of adenosine is the metabolism of ATP to ADP and AMP. AMP is subsequently converted to adenosine by endo- and ecto-5'-nucleotidases (EC 3.1.3.5)(see reviews by Arch & Newsholme, 1978, and Daly, 1982). Adenosine production via this pathway occurs at a much higher rate under conditions of cellular high energy demand (Arch & Newsholme, 1978). Another possible route of adenosine formation involves the enzyme S-adenosylhomocysteinase (EC 3.3.1.1) which cleaves S-adenosylhomocysteine to homocysteine and adenosine (see reviews by Arch & Newsholme, 1978, and Daly, 1982).

Two enzymes involved in the inactivation of adenosine are adenosine deaminase (EC 3.5.4.4) and adenosine kinase (EC 2.7.1.20). Both enzyme systems are located intracellularly and, therefore, adenosine must gain access to the cytoplasm for enzymatic inactivation to occur. Adenosine deaminase converts adenosine to inosine and adenosine kinase catalyses the phosphorylation of adenosine to AMP (Arch & Newsholme, 1978; Daly, 1982). Since the plasma membrane is relatively impermeable to nucleotides, phosphorylation effectively traps nucleosides within cells (see Plunkett & Cohen, 1977). Many studies of adenosine metabolism have been conducted using human erythrocytes as these cells have a rather simple internal environment, due to the absence of mitochondrial and nuclear compartments. In human erythrocytes, it has been determined that adenosine deaminase metabolizes adenosine at a maximal rate (V_{max}) of 200 to 400 nmol/min/ml packed cells (Argarwal *et al.*, 1976) and has a Michaelis Menten constant (K_m) for adenosine of 30 μM (Osborne & Spencer, 1973); adenosine kinase has a V_{max} of 30 to 50 nmol/min/ml packed cells and a K_m for adenosine of 0.7 μM (Kyd & Bagnara, 1980). Using the above values, it can be calculated that adenosine at concentrations greater than 5 μM would preferentially be deaminated to inosine, whereas phosphorylation would predominate at adenosine concentrations less than 3 μM (Hawkins *et al.*, 1979). Therefore, in general, phosphorylation of adenosine predominates at low adenosine concentrations whereas deamination occurs predominantly at higher adenosine concentrations. These two inactivation pathways appear to function together to influence both intracellular adenosine concentrations and the size of the cellular adenine nucleotide pool (see review by Daly, 1982).

The concentration-dependent metabolic fate of adenosine described above has led some investigators to suggest that adenosine kinase may be coupled to the nucleoside transporter in that intracellular phosphorylation facilitates the diffusion of adenosine across plasma membranes (Shimizu *et al.*, 1972). However, the presence of a rapid nucleoside transport mechanism in cells which are deficient in nucleoside kinases (Green, 1980; Plagemann *et al.*, 1981) and the kinetic properties of the enzyme itself (deFazio *et al.*, 1980), suggest that the nucleoside transport system is independent of adenosine kinase and other nucleoside kinases. Similarly, adenosine deaminase has been proposed by Agarwal and Parks (1975) to be closely associated with the nucleoside transporter of erythrocytes. Recent experiments do not support this association and show that the results of Agarwal and Parks (1975) may be explained solely by changes in intracellular adenosine concentration which were produced by their experimental methodologies and the relative K_m and V_{max} values of adenosine kinase and adenosine deaminase (deFazio *et al.*, 1980).

E. Nucleoside Transport

The intracellular location of the enzymes (adenosine deaminase and adenosine kinase) involved in the breakdown of nucleosides suggests that nucleosides must first permeate the cell membrane prior to metabolism. This permeation step is accomplished by a nucleoside specific transport system.

A problem with some studies which attempted to measure rates of nucleoside flux across cell membranes is that they failed to distinguish between nucleoside 'uptake' and nucleoside 'transport'. In this thesis, the term 'uptake' will be used to describe studies which measure cellular accumulation of nucleosides and their metabolic products. This process involves both nucleoside 'transport' and intracellular metabolism of nucleosides. 'Transport' may be considered as the initiating step in the process of nucleoside 'uptake'. The term 'transport' will only be used in this thesis to describe studies which measure 'initial' rates of nucleoside flux. In many cell types, the transport of nucleosides is an extremely rapid process and a steady state of nucleoside flux is attained across the cell membrane within a few seconds (Paterson *et al.*, 1981; Plagemann *et al.*, 1978) and, as a steady state is approached, the rate of nucleoside uptake into the cell becomes similar to the rate of metabolism of 'free' intracellular nucleoside (see Lum *et al.*, 1979). Furthermore, as the intracellular concentrations of influent nucleoside become significant, transporter-mediated back flux of nucleoside must be taken into consideration. Therefore, in order to study nucleoside 'transport' accurately, in cells which have a high transport capacity, nucleoside permeation rates must be measured during the first few seconds after adding the permeant to the system; these time-courses define initial rates, which approximate the rate of nucleoside uptake at 'time zero' upon the addition of nucleoside permeant (Paterson *et al.*, 1981). The driving force for nucleoside transport is the nucleoside concentration gradient across the membrane. This concentration gradient begins to decline immediately upon addition (i.e., 'time zero') of a permeant to the extracellular media. The difficulties associated with the measurement of the extremely rapid rates of adenosine transport may contribute to the discrepancies in the literature regarding the characteristics of adenosine transport. For instance, Lum *et al.*, (1979) described a transport system in P388 cells with a K_m for adenosine of 150 μM whereas Harley *et al.*,(1982), showed that adenosine has a K_m of 15 to 30 μM for the transport systems of a variety of cell types. It should be noted that the methodology used by Lum *et al.*, (1979), in contrast to that used by Harley *et al.*, (1982), did not allow the reliable measurement of transport rates at times of less than 2 seconds which may have resulted in a substantial underestimation of adenosine flux, due to the influence of rapid intracellular adenosine metabolism. Similarly, a recent study by Chello *et al.*, (1983), described two transport mechanisms for adenosine (K_m values of 13 μM and 188 μM at 25°C) in L1210 cells and, as in the study by Lum *et al.*, (1979), these investigators were unable to measure transport rates at times of less than 2 seconds. This limitation could have resulted in artifacts due to rapid adenosine metabolism which may have led Chello *et al.*,(1983), to suggest the presence of 2 distinct adenosine transport systems in L1210 cells. Only those studies which measure rates of adenosine flux using time-courses of less than 2 seconds (see Harley *et al.*, 1982) can be considered as reliable estimations of the characteristics of adenosine transport. The same holds true for transport studies using other nucleosides besides adenosine as well. Relatively non-metabolizable nucleoside permeants such as uridine or thymidine (Oliver & Paterson, 1971; Ungemach & Hegner, 1978; Kessel, 1978) or non-phosphorylating cells (Green, 1980; Plagemann *et al.*, 1981) are often used in these studies in order to eliminate the contribution of intracellular metabolism to the apparent intracellular accumulation of nucleosides.

Nucleoside 'uptake' has been studied in many different biological systems including, among others, cardiac tissue (Olsson *et al.*, 1972), platelets (Sixma *et al.*, 1976), erythrocytes (Schrader *et al.*, 1972; Roos & Pfleger, 1972), pancreatic islets (Welsh & Andersson, 1981), astrocytes (Hertz, 1978), isolated fat cells (Fredholm & Hedqvist, 1979), aortic endothelial and smooth muscle cells (Pearson *et al.*, 1978),

retinal cells (Schaeffer & Anderson, 1981) and cholinergic synapses prepared from *Torpedo* electric organ (Meunier & Morel, 1978). Due to the proposed roles of adenosine in the CNS as a neuromodulator and a regulator of cerebral blood flow, there has been considerable interest in the uptake of adenosine and other nucleosides by CNS tissues. Thymidine uptake has been demonstrated in bovine brain microvessels (Spector & Berlinger, 1982) and a variety of nucleosides are taken up in a carrier-dependent manner by mouse cortical slices (Banay-Schwartz *et al.*, 1980). Adenosine uptake by CNS tissue was first demonstrated by Shimizu *et al.*,(1972), and has since been studied in rat cerebral cortical synaptosomes (Bender *et al.*, 1980; Bender *et al.*, 1981) and isolated rat brain capillaries (Wu & Phillis, 1982b).

Many of the above 'uptake' studies have indicated the presence of more than one uptake system for nucleosides (Sixma *et al.*, 1976; Banay-Schwartz *et al.*, 1980; Bender *et al.*, 1980; Welsh & Andersson, 1981; Schaeffer & Anderson, 1981). For example, two uptake systems for adenosine have been described in human platelets, one with a K_m of 9.8 μM and a 'low-affinity' system with a K_m of 9.4 mM (Sixma *et al.*, 1976). In rat retinal cells, separate uptake systems have been reported for adenosine and guanosine (Schaeffer & Anderson, 1981). These studies must be interpreted with caution as the contributions of passive diffusion and intracellular metabolism to the uptake processes have not been entirely delineated in many cases. Adenosine also appears to accumulate in rat cortical membranes by way of two independent processes, with K_m values for adenosine of 1 μM and 5 μM (Bender *et al.*, 1980). However, in this study it was suggested that the lower affinity system may be located in glial cell membranes.

Initial rate nucleoside 'transport' studies, as defined above, have been performed using mostly isolated cell preparations including various cultured cells (Harley *et al.*, 1982), erythrocytes (Oliver & Paterson, 1971), lymphocytes (Wierda & Pazdernik, 1981), and mouse leukemias (Kessel, 1978; Lum *et al.*, 1979; Chello *et al.*, 1983). However, a recent study has investigated adenosine transport into guinea pig cortical synaptosomes using methodologies approaching those suitable for the measurement of initial rates (Barberis *et al.*, 1981) and these investigators described one high affinity transport system for adenosine with a K_m of 21 µM and a V_{max} of 257 pmol/min/mg protein. Many studies of nucleoside transport have involved the use of erythrocytes which are easily obtained and prepared as homogeneous cell preparations. The transport of nucleosides by human erythrocytes displays kinetic characteristics which are compatible with a simple carrier-mediated system (Wilbrandt & Rosenberg, 1961; Oliver & Paterson, 1971; Cabantchik & Ginsburg, 1977; Paterson *et al.*, 1981). Nucleoside transport is reversible, saturable, non-concentrative (Oliver & Paterson, 1971; Cass & Paterson, 1972; Cabantchik & Ginsburg, 1977; Plagemann *et al.*, 1978) and can be inhibited by a variety of compounds such as dipyridamole and NBMPR (Paterson *et al.*, 1980; Plagemann *et al.*, 1981). The transport system displays a broad specificity in that it accepts as substrates a variety of purine and pyrimidine nucleosides (Oliver & Paterson, 1971; Berlin & Oliver, 1975). Adenosine appears to be the preferred substrate with a K_m value of 1.4 µM for the nucleoside transport system of human erythrocytes (Kolassa *et al.*, 1978a).

Recent studies have indicated that two distinct types of nucleoside transport systems may coexist in human erythrocytes (Kolassa *et al.*, 1978a), mouse lymphocytes (Strauss *et al.*, 1977), rat hepatocytes (Ungemach & Hegner, 1978), and mouse L1210 cells (Chello *et al.*, 1983). In L1210 cells in culture, the 'high affinity' transport system has a K_m for adenosine of 13.9 μM and the 'low affinity' system displays a K_m of 199 μM (Chello *et al.*, 1983); both systems are inhibitable by NBMPR or dipyridamole (Cass *et al.*, 1974; Paterson *et al.*, 1977b; Kolassa *et al.*, 1978a; Chello *et al.*, 1983).

The transport of nucleosides is temperature dependent in that the K_m and V_{max} of adenosine transport (both the 'high' and 'low' affinity systems in human erythrocytes) increase with temperature (Berlin, 1973; Kolassa *et al.*, 1978a), as does the K_m and V_{max} of thymidine transport in cultured cells (Wohlhueter *et al.*, 1979). Also, the K_m and V_{max} of adenosine transport in human erythrocytes decrease 3- to 5-fold with an increase in pH from 7 to 8. However, when using adenosine concentrations less than 2 μM, the decrease in V_{max} of the 'high affinity' transport system (with increasing pH) offsets the concomitant increase in transporter affinity for adenosine and no overall change in adenosine uptake occurs (Kolassa *et al.*, 1978a). At higher adenosine concentrations, a significantly greater rate of adenosine uptake was observed when the pH was raised from 7.4 to 8 due to: i) an increase in V_{max} of the 'low affinity' transport system, and ii) an increase in transporter affinity for adenosine (Kolassa *et al.*, 1978a).

F. Effect of Nucleoside Transport on the Apparent Potency of Adenosine Receptor Agonists and Antagonists

The presence of a specific uptake system which removes an agonist from the vicinity of its receptors may complicate the interpretation of studies involving receptor agonists and antagonsits (Furchgott, 1972; Kenakin, 1980; Kenakin & Beek, 1981). This point is exemplified in a study by Muller and Paton (1979) who showed that 2-chloroadenosine appeared to be a much more potent adenosine receptor agonist than adenosine. As the inclusion of a nucleoside transport inhibitor (hydroxynitrobenzylthioguanosine) and an adenosine deaminase inhibitor (2'-deoxycoformycin) in the assay system decreased the potency difference, they hypothesized that some of this potency difference was due to the rapid uptake and subsequent deamination of adenosine, but not of 2-chloroadenosine. Furthermore, in accordance with proposals of Furchgott (1972) concerning the potency of antagonists for adrenoceptors, theophylline, an adenosine receptor antagonist, had a lower apparent potency as an antagonist of the effects of adenosine than of 2-chloroadenosine (Clanachan & Muller, 1980). This potency difference was reduced in the presence of the nucleoside transport inhibitor, hydroxynitrobenzylthioguanosine, suggesting that the difference was at least partly due to the influence of cellular uptake of adenosine (Clanachan & Muller, 1980)

G. Inhibitors of Nucleoside Transport

As uptake into cells is considered to be an important initial step in the inactivation of adenosine, agents which inhibit the transport mechanism would be expected to potentiate the extracellular actions of both exogenous and endogenous adenosine. Many cytotoxic nucleoside analogues have been synthesized which must enter the cell prior to exerting their biological activity. As with physiological nucleosides, these nucleoside analogues appear to gain access to the cytoplasm via the nucleoside transport system(s). Cells which lack functional nucleoside transporters are resistant to the cytotoxic effects of many of these agents (Cohen *et al.*, 1979; Sherwood & Stollar, 1982). Similarly, inhibitors of nucleoside transport protect cells from the cytotoxic effects of these nucleoside analogues (Paterson *et al.*, 1979a; Uehara & Rabinovitz, 1981).

A novel series of compounds, found to be specific nucleoside transport inhibitors, have been synthesized; these include the 6-thiopurine nucleoside derivatives such as NBMPR (see review by Henderson *et al.*, 1972; Paul *et al.*, 1975). On the other hand, there are agents such as dipyridamole, which were introduced as coronary vasodilators (Kinsella *et al.*, 1962), that have subsequently had their effects attributed to an inhibition of nucleoside transport (Kolassa *et al.*, 1970; Sano, 1974).

Many other pharmacologically active compounds, although having their primary effects on other systems, may also produce some of their effects via an inhibition of nucleoside transport. These agents include prostaglandins (Bender *et al.*, 1982), methylxanthine derivatives (Porsche, 1982), non-steroidal anti-inflammatory drugs (Phillis &

Wu, 1981a), some antibiotics (Choudhury *et al.*, 1982), phenothiazines (Phillis & Wu, 1981b) and benzodiazepines (Phillis *et al.*, 1981). Of these compounds, the benzodiazepines have been the most extensively studied with respect to their influence on adenosine accumulation into cells.

1) 6-Thiopurine nucleoside derivatives:

Analogues of 6-thiopurines were developed during an extensive search for compounds which affect nucleoside metabolism in the hope that some would be useful in the chemotherapy of cancer, or would provide cellular protection against cytotoxic nucleoside analogues (Henderson *et al.*, 1972). Several were found to be potent inhibitors of nucleoside transport (Paterson & Oliver, 1971; Paul *et al.*, 1975; Rogler-Brown & Parks, 1980), the structures of which are shown in Appendix 1. NBMPR, and its water-soluble derivative, nitrobenzylthioinosine 5'-monophosphate, have been the most extensively studied of this group of compounds. NBMPR protects various cultured cells against the cytotoxic effects of several nucleoside analogues including cytosine arabinoside, trifluorothymidine, and 6-azauridine (Paterson *et al.*, 1979b). Similarly, nitrobenzylthioinosine 5'-monophosphate protects mice from potentially lethal doses of tubercidin (Kolassa *et al.*, 1982) and nebularine (Paterson *et al.*, 1979a). Another 6-thiopurine derivative, hydroxynitrobenzylthioguanosine, protects murine L1210 cells from the cytotoxic effects of showdomycin (Uehara & Rabinovitz, 1981). NBMPR and cogeners do not appear to interfere with intracellular nucleoside metabolism since: (i) the transport of nonmetabolizable permeants, such as uridine and thymidine, by human erythrocytes is inhibited by NBMPR (Cass & Paterson, 1972), and

(ii) NBMPR and cogeners have no effect on nucleoside phosphorylation reactions (Olsson *et al.*, 1972; Cass & Paterson, 1977; Paterson *et al.*, 1977b). Both the 'high' and 'low' affinity nucleoside transport systems appear to be inhibited by NBMPR (Kolassa *et al.*, 1978a; Chello *et al.*, 1983). The high potency of NBMPR as a nucleoside transport inhibitor and the rapidity with which it produces this effect has made NBMPR, and cogeners, very useful as "stopping reagents" in initial rate studies of nucleoside transport (Pickard & Paterson, 1972). Radiolabelled NBMPR has also been used as a high affinity probe for the study of the nucleoside transport system (*vide infra*).

2) Coronary vasodilators:

The development of agents with the ability to increase coronary blood flow has been of primary concern to many investigators interested in the treatment of ischaemic heart diseases. Several long-acting vasoactive drugs including dipyridamole, dilazep, hexobendine, lidoflazine, and papaverine, which are structurally unrelated to purines (See Appendix 1), have been reported to potentiate the actions of adenosine and to inhibit the cellular accumulation of adenosine in various experimental preparations.

Dipyridamole, the most extensively studied coronary vasodilator, potentiates adenosine-induced increases in coronary blood flow in dogs (Afonso, 1970; Sano, 1974), cats (Stafford, 1966), and man (Kinsella *et al.*, 1962; Feldman *et al.*, 1981). Dipyridamole alone also produces an increase in coronary blood flow probably by potentiating the vasodilatory effect of endogenous adenosine (Feldman *et al.*, 1981). Adenosine-induced relaxation of bovine coronary arteries could be potentiated by dipyridamole (Kalsner, 1975), as could the negative chronotropic and inotropic effects of adenosine in guinea pig heart (Stafford, 1966; Hopkins, 1973; Kucukhuseyin & Kaysalp, 1974). Other pharmacological responses to adenosine which are potentiated by dipyridamole include relaxation of the smooth muscle of ileum and trachea (Stafford, 1966; Coleman, 1976; Davies *et al.*, 1982), depression of nerve cell firing rates (Phillis *et al.*, 1979b), and adenosine-elicited accumulation of cyclic AMP in guinea pig ventricular muscle strips (Huang & Drummond, 1976) and brain slices (Huang & Daly, 1974; Nimit *et al.*, 1981; Davies *et al.*, 1982). All these effects of dipyridamole can be abolished by adenosine receptor antagonists (Afonso, 1970; Huang & Drummond, 1976; Nimit *et al.*, 1981) which supports the theory that dipyridamole may be producing an increase in the extracellular concentration of adenosine available to interact with adenosine receptors by inhibiting adenosine uptake into cells.

As dipyridamole is a poor inhibitor ($K_i > 100$ μM) of adenosine deaminase (Kubler *et al.*, 1970; Schrader *et al.*, 1972) and adenosine kinase (Schrader *et al.*, 1972), the adenosine-potentiating action of dipyridamole was attributed to an inhibition of the cellular uptake of adenosine (Stafford, 1966; Hopkins, 1973; Colman, 1976; Huang & Drummond, 1976). Inhibition of nucleoside uptake by dipyridamole has been described in a variety of cell types including erythrocytes (Roos & Pfleger, 1972; Turnheim *et al.*, 1978; Rogler-Brown & Parks, 1980; Jarvis *et al.*, 1982b), lymphocytes (Pazdur *et al.*, 1980), platelets (Lips *et al.*, 1980), chick fibroblasts (Scholtissek, 1968), cardiac tissue (Kolassa *et al.*, 1970; Olsson *et al.*, 1972; Mustafa, 1979), Novikoff rat hepatoma cells (Plagemann, 1971), guinea pig smooth muscle (Kolassa *et al.*, 1978b), cholinergic synaptosomes from

Torpedo electric organ (Meunier & Morel, 1978), guinea pig and rat cortical synaptosomes (Bender *et al.*, 1980; Barberis *et al.*, 1981), and rat brain capillaries (Wu & Phillis, 1982b).

The mechanism by which dipyridamole produces its inhibition of nucleoside uptake is a matter of some dispute. Strictly competitive inhibition has been observed in nucleoside-permeable sheep erythrocytes (Jarvis *et al.*, 1982b), platelets (Lips *et al.*, 1980), chick fibroblasts (Scholtissek, 1968), and rat Novikoff hepatoma cells (Plagemann, 1971). However, other investigators found either a mixed (Turnheim *et al.*, 1978), or non-competitive (Meunier & Morel, 1978; Rogler-Brown & Parks, 1980) type of inhibition. Furthermore, a wide range of inhibition constants (K_i values of 0.02 μM to 47 μM) for dipyridamole have been reported (Scholtissek, 1968; Plagemann, 1971; Meunier & Morel, 1978; Turnheim *et al.*, 1978; Rogler-Brown & Parks, 1980; Lips *et al.*, 1980; Jarvis *et al.*, 1982b). Some of these discrepancies may be due to methodological differences among the studies.

Dipyridamole appears to be a relatively non-specific inhibitor of membrane transport processes because it also inhibits the transport of anions, purine bases, 2-deoxy-D-glucose, and D-glucosamine (Plagemann & Richey, 1974). Binding of dipyridamole to sites relevant to these latter processes may result in a significant reduction in the amount of dipyridamole available to interact with the nucleoside transport system in test systems and this depletion effect may explain some of the confusion over the nature and potency of nucleoside transport inhibition produced by dipyridamole. However, in many cases, no adequate explanation has been proposed for the wide variation in the nature of inhibition (competitive, noncompetitive, mixed) by dipyridamole nor the wide range of K_i values observed. Several studies also suggest that there may be significant species variation in the potency of dipyridamole as an inhibitor of nucleoside transport (Kolassa *et al.*, 1971; Hopkins & Goldie, 1971; Sakai *et al.*, 1981; Wu *et al.*, 1981; Barker & Clanachan, 1982).

Dilazep and the structurally related compound hexobendine (see Appendix 1) have been shown to increase coronary (Buyniski *et al.*, 1972; Sano, 1974; Marshall & Parratt, 1974; Fujii *et al.*, 1981) and cerebral (Buyniski *et al.*, 1972) blood flow in dogs. Both enhance the coronary vasodilator effect of adenosine in dogs (Raberger & Kraupp, 1971). Hexobendine also potentiates adenosine-induced accumulation of cyclic AMP in ventricular myocardium (Huang & Drummond, 1976) and in brain slices (Huang & Daly, 1974). Both hexobendine and dilazep have been shown to augment the negative inotropic and chronotropic effects of adenosine (Hopkins, 1973; Fujita *et al.*, 1980). These coronary vasodilators are also potent inhibitors of adenosine uptake (K_i values less than 0.1 μM) in human erythrocytes (Turnheim *et al.*, 1978), cardiac cells (Mustafa, 1979), guinea pig epithilium (Kolassa *et al.*, 1978b), and rat brain capillaries (Wu & Phillis, 1982b). In many of these studies, the potencies of dilazep and hexobendine may have been underestimated as both of these compounds are cleaved quite rapidly by ester hydrolases (Schriewer & Rauen, 1972; Schaumloffel & Clausnitzer, 1972; Kolassa *et al.*, 1977; Kolassa *et al.*, 1978c) which may be present in isolated tissue and membrane preparations.

Lidoflazine is a long-acting coronary vasodilator (Schaper *et al.*, 1966) which acts both directly on the smooth muscle of coronary vessels (Schaper *et al.*, 1966; VanNeuten & Wellens, 1979; VanNeuten & Vanhoutte, 1980) and indirectly via a potentiation of adenosine-induced vasodilation (Afonso *et al.*, 1968; Jageneau & Schaper, 1969; Van Belle, 1970a). The direct effect of lidoflazine appears to be due to an inhibition of calcium influx (VanNeuten & Wellens, 1979; VanNeuten & Vanhoutte, 1980) thereby inhibiting smooth muscle contraction. Adenosine-induced vasodilation in dogs is significantly potentiated by lidoflazine (Afonso *et al.*, 1968; Jageneau & Schaper, 1969) as are the negative inotropic and chronotropic effects of adenosine (Kucukhuseyin & Kaysalp, 1974). Lidoflazine decreases the rate of disappearance of adenosine from whole blood of various species (Van Belle, 1970a; Van Belle, 1970b), and these effects were attributed to an inhibition of adenosine uptake (Van Belle, 1970a; Kucukhuseyin & Kaysalp, 1974). Lidoflazine also inhibits the uptake of adenosine by cardiac cells (Mustafa, 1979) and guinea pig epithelial cells (Kolassa *et al.*, 1978b). However, lidoflazine was generally 10-fold less potent than dipyridamole as an inhibitor of adenosine uptake (Mustafa, 1979).

Papaverine, a naturally occurring alkaloid isolated from the opium poppy (*Papaver somiferum*), causes smooth muscle relaxation by a mechanism which is generally considered to involve an inhibition of cyclic AMP phosphodiesterases thereby resulting in elevated intracellular cyclic AMP levels (Kukovetz & Poch, 1970; Poch & Kukovetz, 1971). It also potentiates adenosine-elicited accumulation of cyclic AMP in brain slices (Huang & Daly, 1974). Recent reports, however, indicate that papaverine is also a weak inhibitor (relative to dipyridamole, dilazep and hexobendine) of adenosine uptake into astrocytes (Hertz, 1978), rabbit pulmonary arterial tissue (Katsuragi & Su, 1982b), cardiac cells (Mustafa, 1979), and rat brain capillaries (Wu & Phillis, 1982b). Therefore, it is possible that potentiation of adenosine effects may contribute to smooth muscle relaxation produced by high concentrations of papaverine.

In general, the order of potency of the above mentioned coronary vasodilators as adenosine uptake inhibitors is dipyridamole > dilazep > hexobendine > lidoflazine > papaverine with IC_{50} values for inhibition of adenosine uptake by cardiac cells ranging from 40 nM for dipyridamole to 20 μM for papaverine (Mustafa, 1979).

3) Benzodiazepines:

a) Pharmacological effects and mode of action: Diazepam and other benzodiazepines (see Appendix 1 for representative structures) have wide therapeutic application as anxiolytics, anticonvulsants, and muscle relaxants. Diazepam is also commonly used in anaesthetic practice and has been advocated for the induction of anaesthesia. The exact mode of action of the benzodiazepines remains obscure. However, at least some of their effects may be explained by a potentiation of *gamma*-aminobutyric acid inhibitory neurotransmission in the CNS (see review by Gallager, 1982). Several different types of benzodiazepine recognition sites have been characterized in both neuronal and non-neuronal tissues. It has been proposed that the benzodiazepines are producing their effects via interactions with some or all of these sites (see reviews by Tallman *et al.*, 1980, and Gallager, 1982).

b) Characteristics of benzodiazepine recognition sites:

(i) 'Neuronal' benzodiazepine recognition sites: High-affinity 'neuronal' benzodiazepine recognition sites were characterized using [^{3}H]diazepam or [^{3}H]flunitrazepam in membranes prepared from rat brain (Braestrup & Squires, 1977; Mohler & Okada, 1977a; Mohler & Okada, 1977b; Squires & Braestrup, 1977). Subsequently, [^{3}H]diazepam was found to bind in a similar manner to membranes prepared from human brain tissue (Mohler & Okada, 1978). The neuronal localization of these benzodiazepine sites (Mohler & Okada, 1977b; Bosmann *et al.*, 1978) supports the proposal that benzodiazepines act as modulators of neuronal activity (Gallager, 1982). Furthermore, the order of affinity of a series of benzodiazepines for the high affinity 'neuronal' benzodiazepine recognition sites closely correlates with their order of potency as anxiolytics (Mohler & Okada, 1978) and muscle relaxants (Mohler & Okada, 1977b). Benzodiazepine recognition sites can be labelled *in vivo* following parenteral administration of either [^{3}H]diazepam or [^{3}H]flunitrazepam. Studies of this type indicate an excellent correlation between benzodiazepine recognition site occupancy *in vivo* and the degree of protection against pentylenetetrazol- or picrotoxin-induced seizures (Duka *et al.*, 1979; Paul *et al.*, 1979). Therefore, since binding can be directly correlated with a pharmacological effect, these high affinity 'neuronal' benzodiazepine recognition sites have been classified as benzodiazepine 'receptors'. Further evidence for the pharmacological relevance of these sites comes from the existence of specific benzodiazepine antagonists (see review by Mohler & Richards, 1981). Ro 15-1788 and CGS 8216 are potent antagonists of many centrally-mediated benzodiazepine effects (Hunkeler *et al.*, 1981; Mohler *et al.*, 1981; Polc *et al.*, 1981b; Czernik *et al.*, 1982) and these compounds are potent inhibitors of [^{3}H]diazepam and [^{3}H]flunitrazepam binding to high affinity 'neuronal' benzodiazepine recognition sites (Mohler *et al.*, 1981; Mohler & Richards, 1981; Czernik *et al.*, 1982). Ro 15-1788 will also precipitate a benzodiazepine withdrawal reaction in baboons after long-term (45 days) administration of diazepam (Lukas & Griffiths, 1982), as would be expected from a specific receptor antagonist.

The existence of these high affinity, specific recognition sites for benzodiazepines has suggested the existence of endogenous benzodiazepine-like ligands. This situation is analogous to the characterization of an opiate recognition site and the subsequent search for the endogenous opiate-like compounds (see review by Szara, 1982). Many different substances have been reported, by various research groups, to represent the endogenous ligand for the 'neuronal' benzodiazepine sites in the CNS (see review by Mohler, 1981). The list of proposed endogenous ligands includes purines (Damm *et al.*, 1979; Marangos *et al.*, 1979), nicotinamide (Mohler *et al.*, 1979), thromboxane A_2 (Allay *et al.*, 1978), ethyl β-carboline-3-carboxylate (Braestrup *et al* ., 1980), various peptides, and proteins (Davis & Cohen, 1980; Chiu & Rosenberg, 1981; Kenessey *et al.*, 1981; Woolf & Nixon, 1981); of these, ethyl β-carboline-3-carboxylate displayed the highest affinity for the 'neuronal' benzodiazepine site (Braestrup *et al.*, 1980). It has since been shown, however, that ethyl β-carboline-3-carboxylate was probably generated during the isolation procedure and is not an endogenous compound (Nielsen *et al.*, 1981). This, however, does not negate the proposal that an endogenous compound containing a β-carboline nucleus may exist as a benzodiazepine receptor ligand. In support of this proposal are studies which show that a wide variety of β-carboline derivatives have affinity for the high affinity 'neuronal' benzodiazepine site (Robertson *et al.*, 1981; Cain *et al.*, 1982).

(ii) Heterogeneity of 'neuronal' benzodiazepine recognition sites: It seems unlikely that the benzodiazepines are producing their multiple pharmacological effects through a single receptor mechanism although it is possible that one receptor type mediates different responses in different brain areas (Robertson, 1980). Structure-activity studies with benzodiazepines show that the structural requirements for sedative effects differ from those necessary for anxiolytic effects, suggesting that different receptors mediate these two actions of the benzodiazepines (Babbini *et al.*, 1979). A substantial amount of pharmacological and biochemical evidence has been compiled in support of the existence of multiple types of benzodiazepine recognition sites located on neural elements in CNS membranes (Hirsch *et al.*, 1982; Hunt, 1982; Lippa *et al.*, 1982; Volicer & Biagioni, 1982). For example, one type of benzodiazepine site displays a high affinity for both benzodiazepines and triazolopyridazines, is not coupled to *gamma*-aminobutyric acid receptors or chloride ionophores, and appears to be involved in the anxiolytic effects of benzodiazepines. Another type of site has high affinity for benzodiazepines, but low affinity for triazolopyridazines, is coupled to *gamma*-aminobutyric acid receptors and/or chloride ionophores and mediates sedation and ataxia (Klepner *et al.*, 1979). These two receptor types have different regional distributions

(Klepner *et al.*, 1979) and can be isolated as different proteins by electrophoretic techniques (Sieghart & Karobath, 1980).

Another possible explanation for apparent receptor heterogeneity, rather than invoking independent binding sites, is two interconvertible conformations of a single site. Evidence for multiple conformations of benzodiazepine binding sites comes from the demonstration that flunitrazepam exhibits biphasic association and dissociation kinetics with its binding sites and that the dissociation rates vary with incubation time, temperature and *gamma*-aminobutyric acid concentration (Chiu *et al.*, 1982; Quast *et al.*, 1982). Quast and Maklmann (1982) have recently reported that flunitrazepam can induce a conformation change in the benzodiazepine receptor complex and Karobath and Supavilai (1982) have suggested that the benzodiazepine receptor complex consists of four benzodiazepine binding sites where irreversible binding of flunitrazepam to one of the sites affects the affinity of the three remaining sites for benzodiazepine agonists but not for benzodiazepine antagonists.

Other types of benzodiazepine recognition sites which are distinct from the 'neuronal' sites described above also exist in CNS and peripheral tissues.

(iii) 'Non-neuronal' benzodiazepine recognition sites: Benzodiazepine recognition sites, which are distinct from 'neuronal' benzodiazepine binding sites, exist in 'non-neuronal' elements of CNS membrane preparations (Gallager *et al.*, 1981), as well as in various peripheral membranes such as platelets (Wang *et al.*, 1980), mast cells (Taniguchi *et al.*, 1980), and membranes prepared from the heart and kidney (Syapin & Skolnick, 1979; Davies & Huston, 1981; Taniguchi *et al.*, 1982). These 'non-neuronal' benzodiazepine sites have a high affinity for the benzodiazepine derivative Ro 5-4864 and a low affinity for clonazepam. This ligand specificity is opposite to that determined for the 'neuronal' benzodiazepine sites (Gallager *et al.*, 1981). Furthermore, radiation-inactivation analysis indicated that the 'non-neuronal' benzodiazepine site has a molecular weight significantly different from that of the 'neuronal' benzodiazepine site (Paul *et al.*, 1981). A biological effect has yet to be associated with benzodiazepine occupation of this 'non-neuronal' benzodiazepine recognition site.

(iv) 'Low affinity' benzodiazepine recognition sites: Still another type of benzodiazepine recognition site has been identified in rat brain membranes. This site displays micromolar affinities (1000 times lower affinity than the 'neuronal' and 'non-neuronal' benzodiazepine sites) for benzodiazepines and appears to have characteristics distinct from both the 'neuronal' and the 'non-neuronal' benzodiazepine sites (Bowling & DeLorenzo, 1982).

From the preceding account, it is apparent that many different types of benzodiazepine recognition sites are present in both peripheral and central mammalian tissues some of which have yet to be linked to a pharmacological effect of the benzodiazepines. It is unlikely that all of these sites are involved in a potentiation of *gamma*-aminobutyric acid inhibitory neurotransmission.

c) Benzodiazepine-nucleoside interactions: Several lines of evidence have suggested a relationship between the actions of benzodiazepines and purine nucleosides.

(i) Compounds of both classes produce similar behavioural effects including sedation, hypnosis, and anticonvulsant activity (Crawley *et al.*, 1981b; Yarbrough & McGuffin-Clineschmidt, 1981; Dunwiddie & Worth, 1982).

(ii) The purines inosine and hypoxanthine have been proposed as endogenous ligands for the benzodiazepine receptor (Skolnick *et al.*, 1978; Skolnick *et al.*, 1980c). Inosine and hypoxanthine, although displaying very low affinity for the 'neuronal' benzodiazepine site (≃1 mM), may reach sufficient concentrations in the CNS, at times of high neuronal activity, to produce effects via benzodiazepine site interactions (Skolnick *et al.*, 1980c).

(iii) Inosine antagonizes pentylenetetrazole-evoked seizures, a model system for assessing benzodiazepine-like activity (Skolnick *et al.*, 1979), and inosine, like adenosine and adenosine analogues, reverses diazepam-induced stimulation of exploratory behaviour in mice (Crawley *et al.*, 1981a; Crawley *et al.*, 1981b). The latter result suggests an antagonistic interaction between purines and benzodiazepines with respect to their effect on experimental models of anxiety (Crawley *et al.*, 1981a).

(iv) Electroshock-seizures in mice result in an increase in inosine and hypoxanthine concentrations in the brain and these high purine levels are believed to terminate the seizure activity possibly by interacting with benzodiazepine receptors (Lewin & Bleck, 1981); it was proposed from these studies that inosine and hypoxanthine may function as endogenous antiepileptic agents (Lewin & Bleck, 1981).

(v) Various purine analogues, synthetic or isolated from natural sources, have affinity for the 'neuronal' benzodiazepine recognition sites, as shown by inhibition of [^{3}H]diazepam binding (Davies *et al.*, 1980a; Sung & Saneyoshi, 1982). The most potent of these are the naturally occuring purine, 1-methylisoguanosine, with a K_i of 15 μM (Davies *et al.*, 1980a) and the synthetic purine, 6-methylthioguanine, with a K_i of 16 μM (Sung & Saneyoshi, 1982); both of these agents are about 30-fold more potent than inosine and hypoxanthine.

(vi) Several purine-like compounds have been developed which show high affinity ($IC_{50} < 100$ nM) for the 'neuronal' benzodiazepine binding site; these include triazolopyridazines (Squires *et al.*, 1979), imidazolopyrimidines, pyrrolopyrizine, and zopiclone (Marangos *et al.*, 1979). EMD 28422, a purine derivative with pharmacological effects similar to the benzodiazepines (Skolnick *et al.*, 1980a), appears to cause an increase in the number of 'neuronal' benzodiazepine binding sites without altering the affinity of diazepam for the sites (Skolnick *et al.*, 1980b).

(vii) Nucleoside transport inhibitors such as dipyridamole also inhibit the binding of [^{3}H]diazepam to both 'neuronal' (Davies *et al.*, 1980a; Wu *et al.*, 1980) and 'non-neuronal' (Davies & Huston, 1981) benzodiazepine binding sites. However, transport inhibitors are effective in this regard only at concentrations which are higher than those required for the inhibition of nucleoside transport.

(viii) Caffeine, which is a phosphodiesterase inhibitor and an adenosine receptor antagonist, produces characteristic CNS-mediated stimulant effects and, in high doses, has been reported to cause seizures in experimental animals (Ritchie, 1975). Benzodiazepines reverse these actions of caffeine(Marangos *et al.*, 1981; DeAngelis *et al.*, 1982), which suggests that caffeine may cause seizures by interacting with a benzodiazepine recognition site at which the benzodiazepines act as antagonists. In support of this hypothesis, caffeine and various other methylxanthines inhibit the binding of [^{3}H]diazepam to the 'neuronal' benzodiazepine recognition sites (Marangos *et al.*, 1979). Caffeine reverses the effects of diazepam in cats (Polc *et al.*, 1981a) and in man (Mattila *et al.*, 1982). Aminophylline, also an adenosine receptor antagonist, reverses the sedative effects of diazepam in man (Stirt, 1981). It should be noted that the doses of caffeine and aminophylline used in many of these studies result in blood concentrations which are higher than those produced by average coffee consumption or normal clinical doses. Since these agents are CNS stimulants, the interaction with benzodiazepines may simply represent physiological antagonism.

Nevertheless, the extensive evidence supporting benzodiazepine-purine interactions in the CNS strongly suggests that some of the effects of the benzodiazepines may involve purinergic mechanisms.

d) Effect of benzodiazepines on adenosine uptake: A benzodiazepine-purine interaction which has recently received considerable attention has been the ability of benzodiazepines to inhibit the cellular accumulation of adenosine. Indeed, potentiation of the effects of adenosine via an inhibition of adenosine uptake has been proposed as a means by which benzodiazepines may produce some of their clinical effects (Wu *et al.*, 1981).

Cardiovascular depression has long been recognized as a significant side-effect of diazepam administration (Rolly, 1976). Diazepam causes vasodilation of coronary and systemic blood vessels in dogs (Abel *et al.*, 1970), and increases coronary blood flow in dogs (Daniell, 1975), and in man (Ikram *et al.*, 1973). These effects of diazepam are possibly mediated through a potentiation of endogenous adenosine since adenosine-induced coronary vasodilation in anaesthetized dogs and adenosine-induced depression of guinea-pig left atria contractions are potentiated by diazepam (Clanachan & Marshall, 1980a; Clanachan & Marshall, 1980b; Kenakin, 1982). Diazepam also potentiates the effects of adenosine on various smooth muscle preparations (Clanachan & Marshall, 1980b; Clanachan & Deboer, 1980; Slater & Bennett, 1982) as well as adenosine-mediated neuronal depression in the CNS (Phillis, 1979). Furthermore, theophylline, an adenosine receptor antagonist, blocks flurazepam-induced depression of the firing of cerebral cortical neurones (Phillis *et al.*, 1979a). The inability of diazepam to enhance the effects of 2-chloroadenosine (Clanachan & Marshall, 1980b), an adenosine analogue, the effects of which are not potentiated by the recognized nucleoside transport inhibitor hydroxynitrobenzylthioguanosine and, therefore, is not considered to be a permeant of the transport system (Muller & Paton, 1979), suggests that potentiation of adenosine effects by diazepam and other benzodiazepines may be attributed to inhibition of the nucleoside transport system with a consequent decrease in cellular uptake and metabolism of adenosine.

Inhibition of adenosine uptake by benzodiazepines has been demonstrated in guinea pig brain slices (Mah & Daly, 1976; Traversa &

Newman, 1979; York & Davies, 1982), rat cortical synaptosomes (Phillis *et al.*, 1981), guinea pig cardiac muscle (Barker & Clanachan, 1982), and rabbit pulmonary arterial tissue (Katsuragi & Su, 1982b). The order of potency of the benzodiazepines as adenosine uptake inhibitors (determined as IC_{20} values = concentration required to inhibit uptake by 20%) in rat cortical synaptosomes is similar to their anxiolytic order of potency (Phillis *et al.*, 1981). However, in all systems studied, benzodiazepine concentrations greater than 10 μM were required to produce a 50% inhibition of adenosine uptake (Mah & Daly, 1976; Traversa & Newman, 1979; Phillis *et al.*, 1981; Barker & Clanachan, 1982; York & Davies, 1982). In rabbit pulmonary arterial segments, diazepam was a weaker inhibitor of adenosine uptake than papaverine, the least potent of the adenosine uptake inhibitors described in previous sections (Katsuragi & Su, 1982b).

The close correlation between the ability of benzodiazepines to inhibit adenosine uptake and their anxiolytic order of potency, and the finding that nucleoside transport inhibitors, such as hexobendine, inhibit the binding of [^{3}H]diazepam to rat brain cortical membranes (Wu *et al.*, 1980), has led Phillis and coworkers to suggest that the 'neuronal' benzodiazepine recognition sites and the CNS nucleoside transport system(s) are functionally coupled (Wu *et al.*, 1981). However, due to the relatively long incubation times used (> 25 s) in all of the uptake studies (Mah & Daly, 1976; Traversa & Newman, 1979; Phillis *et al.*, 1981; Barker & Clanachan, 1982; Katsuragi & Su, 1982b; York & Davies, 1982), adenosine 'uptake' and not adenosine 'transport' was measured. Therefore, it cannot be stated conclusively that the benzodiazepines are producing effects via an inhibition of nucleoside transport, rather than affecting some other aspect of nucleoside metabolism.

H. Use of [^{3}H]Nitrobenzylthioinosine as a Probe for the Nucleoside Transport System

A major problem associated with nucleoside flux studies has been the development of technologies required to measure the extremely rapid rates of nucleoside transport. Changes with time in the cell content of isotope derived from exposure to radiolabelled adenosine may reflect entry by passive diffusion, metabolic trapping by conversion of adenosine to impermeable phosphate esters, and efflux of adenosine, inosine, and hypoxanthine. Initial rates of permeant influx represent unidirectional influx and are those of the first step (transport) in the multistep process of adenosine uptake. Thus, it is critical in the interpretation of permeant uptake rates in terms of transport that those rates be initial rates.

Measurement of initial rates often requires experimental time-courses of less than 5 s. These studies also require intact cells or vesicles which means that harsh separation procedures which may disrupt biological membranes cannot be used to achieve rapid termination of the assays. To circumvent these problems, 'transport' methodologies are necessarily complex and true initial rate transport studies have yet to be performed in fragile systems such as synaptosomes.

An alternate way of studying the nucleoside transport system is with the use of [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR) as a ligand. Radiolabelled NBMPR binds tightly, but reversibly to specific sites in membranes of various cultured cells (Lauzon & Paterson, 1977; Wohlhueter *et al.*, 1978; Paterson *et al.*, 1980; Dahlig-Harley *et al.*, 1981) and erythrocytes (Pickard *et al.*, 1973; Cass *et al.*, 1974; Cass & Paterson, 1976; Jarvis *et al.*, 1982b). More recently, NBMPR binding sites have been demonstrated in rat CNS membranes (Marangos *et al.*, 1982a; Wu & Phillis, 1982a). The affinity of NBMPR for these sites ranges from 0.1 to 1 nM although the number of binding sites (per cell, or per mg protein) varies widely among cell types.

The binding of NBMPR to specific sites in erythrocyte membranes has been directly correlated with inhibition of nucleoside transport in these cells, suggesting that NBMPR interacts specifically with components of the erythrocyte nucleoside transport system (Cass *et al.*, 1974). Binding of NBMPR to specific sites in HeLa cells has also been correlated with inhibition of uridine and thymidine uptake. However, in these cells, a substantial proportion of the total transport capability ($\approx 30\%$) was not inhibited by 5 nM NBMPR, a concentration which saturates the high affinity NBMPR binding sites (Lauzon & Paterson, 1977). A much higher concentration of NBMPR was required to inhibit the remaining transport component (Lauzon & Paterson, 1977; Dahlig-Harley *et al.*, 1981). These latter results suggest the presence of two nucleoside transport systems in HeLa cells which differ in their sensitivity to inhibition by NBMPR. This hypothesis is supported by recent results which describe biphasic concentration-effect relationships for the inhibition of nucleoside transport in HeLa cells by NBMPR (Paterson *et al.*, 1980; Dahlig-Harley *et al.*, 1981). Similarly, a component of nucleoside transport which is relatively insensitive to NBMPR has been demonstrated in several other types of cultured cells including mouse leukemia L1210 cells (Belt, 1983), Novikoff hepatoma cells (Wohlhueter *et al.*, 1979), and a line of cultured cells derived from the Walker 256 carcino-sarcoma (Paterson *et al.*, 1983). The Walker 256 cells also appear to lack specific high affinity binding sites for [^{3}H]NBMPR (Paterson *et al.*, 1983). Therefore, a type of nucleoside transport system seems to exist which does not have nucleoside transport inhibitory [^{3}H]NBMPR binding sites.

However, with one exception known to date (Dr A.R.P. Paterson, personal communication), all high affinity ($K_D < 5$ nM) [^{3}H]NBMPR binding sites so far characterized appear to be associated with functional nucleoside transport systems. Nucleoside-impermeable sheep erythrocytes (Jarvis & Young, 1980) and nucleoside transport-deficient mouse lymphoma cells (Cass *et al.*, 1981) do not possess high affinity [^{3}H]NBMPR binding sites. Furthermore, reticulocytes from a strain of sheep which have nucleoside-impermeable mature erythrocytes, transport nucleosides rapidly and the loss of this transport capability upon maturation correlates with the loss in high affinity [^{3}H]NBMPR binding sites (Jarvis & Young, 1982). A similar situation was observed during the replication cycle of HeLa cells where [^{3}H]NBMPR site density parallels the fluctuations in nucleoside uptake that occur during the cell cycle (Cass *et al.*, 1979). The exception to this direct relationship between NBMPR and functional nucleoside transporters has been found in cells of a virus-transformed clone (Nil SV) derived from Nil 8 hamster fibroblasts. Nil SV cells have about 10^6 binding sites (B_{max}) for [^{3}H]NBMPR, but apparently have the same uridine transport capability (influx V_{max}) as Nil 8 cells which display 10-fold fewer (10^5) binding sites for [^{3}H]NBMPR. This study indicated that Nil SV cells may have specific [^{3}H]NBMPR binding sites which are not associated with functional nucleoside transporters (Dr. A.R.P. Paterson, personal communication).

[^{3}H]NBMPR has recently been used as a tool to investigate the characteristics of the nucleoside transport system of erythrocytes from various species (Jarvis & Young, 1980; Jarvis *et al.*, 1982a). Assuming that, in erythrocytes, each NBMPR molecule binds only to one functional transporter (Jarvis & Young, 1980), it can be determined, from [^{3}H]NBMPR binding constants (B_{max}) and the kinetic constants (V_{max}) of uridine transport, that the apparent translocation capacity of the nucleoside transporter (140 to 180 molecules of uridine/site/s at 25°C; Jarvis *et al.*, 1982a) is similar in erythrocytes from all species so far tested. Therefore, although erythrocytes from different species vary widely in their ability to transport uridine, the capability of a cell to transport uridine is a function of the number of operational nucleoside transporters rather than changes in individual transporter activity (Jarvis *et al.*, 1982a).

The binding of [^{3}H]NBMPR to high affinity sites is inhibited by a variety of structurally unrelated compounds which are also inhibitors of nucleoside transport. Recent reports describe the inhibition by dipyridamole of site-specific binding of [^{3}H]NBMPR to erythrocyte membranes and HeLa cells (Cass & Paterson, 1976; Jarvis & Young, 1980; Paterson *et al.*, 1980). In HeLa cells, it was suggested that dipyridamole may inhibit nucleoside transport by interacting with the same site as NBMPR and has an apparent K_D at this site of 30 nM (Paterson *et al.*, 1980). Similarly, the nucleoside transport inhibitor, lidoflazine, inhibited the site-specific binding of [^{3}H]NBMPR to HeLa cells (Paterson *et al.*, 1980). The apparently competitive inhibition of the binding of [^{3}H]NBMPR by dipyridamole was unexpected considering the obvious structural differences between dipyridamole and NBMPR (Paterson *et al.*, 1980).

The mode of inhibition of nucleoside transport by NBMPR and cogeners is a matter of some controversy. Competitive, partially competitive (mixed), and non-competitive inhibition kinetics have been observed depending on the particular methodology ('uptake' or 'transport'), and the cell type used (Cass & Paterson, 1977; Paterson *et al.*, 1977a; Eilam & Bibi, 1977; Turnheim *et al.*, 1978; Wohlhueter *et al.*, 1978; Cass *et al.*, 1979).

Another matter of confusion is whether or not [^{3}H]NBMPR binds to the permeant site or a separate transport inhibitory site of the nucleoside transport system. A number of nucleosides are competitive inhibitors of the binding of [^{3}H]NBMPR to human erythrocytes (Cass & Paterson, 1976; Paterson, 1979). However, deoxycytidine, also a substrate for the transport system (Cass & Paterson, 1972; Jarvis *et al.*, 1982a), was shown in one study (Cass & Paterson, 1976) to be an ineffective inhibitor of the binding of [^{3}H]NBMPR which suggested that [^{3}H]NBMPR did not bind to the permeant site by instead, bound to a distinct transport inhibitory site. In contrast, a recent study by Jarvis *et al.*, (1982b), showed that deoxycytidine, like other nucleoside permeants, does, indeed, inhibit the binding of [^{3}H]NBMPR in an apparently competitive manner. Furthermore, the K_i values for inhibition of the binding of [^{3}H]NBMPR by uridine are similar to the K_m values for uridine equilibrium exchange influx in erytrocytes (Jarvis *et al.*, 1982a & b). These results are consistent with the view that [^{3}H]NBMPR binds to the permeant site of the nucleoside transport system. Arguements against the proposal that [^{3}H]NBMPR binds to the permeant site of the nucleoside transport system include: 1) the apparent structural disparities between some competitive inhibitors of the binding of [^{3}H]NBMPR (e.g., dipyridamole) and nucleosides, and 2) the demonstration of nucleoside transport in cells which lack [^{3}H]NBMPR binding sites (*vide supra*, and see Paterson *et al.*, 1983). Further experiments need to be performed in order to resolve the exact identity of the [^{3}H]NBMPR binding site.

Detailed studies of uridine influx and efflux in erythrocytes showed that NBMPR was a competitive inhibitor of uridine influx but was a non-competitive inhibitor of uridine efflux from erythrocytes (Jarvis *et al.*, 1982b). These results indicate that NBMPR binding to an external site of the nucleoside transport system inhibits uridine translocation (Deves & Krupta, 1978; Jarvis *et al.*, 1982b).

The nucleoside transport system also exhibits chemical asymmetry in that p-chloromercuriphenylsulphonate and trypsin only disrupted nucleoside transport and high affinity binding of [^{3}H]NBMPR if the inner membrane was exposed to these agents (Jarvis & Young, 1982). The results outlined above (Jarvis & Young, 1982; Jarvis *et al.*, 1982b), along with the finding that uridine can protect the nucleoside transport system against the effects of p-chloromercuriphenylsulphonate (Jarvis & Young, 1982), led these investigators to propose a model of the nucleoside transporter consisting of one substrate binding site and two interconvertible carrier conformations. It was suggested that transport (influx or efflux) depends upon a conformational change in the carrier and that this process occurs more rapidly in the presence

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of substrate than in its absence. In this model, [^{3}H]NBMPR binds to both the permeant site and a hydrophobic site located only on the external surface of the carrier.

The apparent molecular weight of the [^{3}H]NBMPR binding complex of human erythrocytes was estimated by radiation-inactivation analysis, to be 122,000 (Jarvis *et al.*, 1980). A partial purification (13-fold) of this binding complex has been achieved (Jarvis & Young, 1981).

Recent studies have shown that NBMPR can be used as a photo-affinity label (Marangos *et al.*, 1982a; Young *et al.*, 1983) thus providing a means of irreversibly labelling the nucleoside transport complex. This development should greatly facilitate further purification of the NBMPR binding complex and should also lead to a more detailed characterization of the nucleoside transport system.

I. Rationale

The foregoing account has outlined the significance of the nucleoside transport system, and its inhibition by several classes of drugs, in the actions of physiological and synthetic nucleosides in the body. Adenosine, a physiological nucleoside, has been attributed a wide variety of regulatory functions which are mediated through stimulation of extracellular adenosine receptors. These actions of adenosine may be terminated by its uptake into cells followed by enzymatic metabolism.

It has been proposed that several classes of pharmacologically active compounds owe some of their effects to an inhibition of the cellular metabolism of adenosine, possibly due to an inhibition of the membrane transport of adenosine. These drugs include 6-thiopurine nucleoside derivatives (e.g., nitrobenzylthioinosine), coronary vasodilators (e.g., dipyridamole), and benzodiazepines (e.g., diazepam).

Many experimental investigations assessing interactions of drugs with the nucleoside transport system have suffered certain inherent limitations. Observed values of cellular or vesicular uptake of nucleosides have probably represented the net effects of several metabolic processes, in addition to that of membrane transport. Consequently, drug-induced changes in nucleoside uptake have not necessarily been due to changes in nucleoside transporter activity. These problems have probably contributed to the degree of confusion in the literature concerning the nature and potency of transport inhibitors in various experimental systems.

The general objective of the present study is to provide a clearer understanding of drug interactions with the nucleoside transport system. In order to avoid the problems associated with nucleoside flux studies, the current experiments utilize the probe, NBMPR.

From the review of previous experimental studies with [^{3}H]NBMPR, it is apparent that the most extensive information has been obtained with erythrocytes or cultured cells. In human erythrocytes, nucleoside transporter activity ceases when specific membrane sites are occupied by NBMPR, or certain cogeners. [^{3}H]NBMPR binds tightly, but reversibly, to these transport inhibitory sites, and inhibition of transport is related to site occupancy. Furthermore, [^{3}H]NBMPR binding sites appear to be present only on elements of functional nucleoside transporters in erythrocytes.

Consequently, erythrocytes appear to provide a convenient system for the characterization of drug interactions with the nucleoside transport system. Such a characterization might involve:

1) measurement of the affinities of several classes of nucleoside transport inhibitors for [^{3}H]NBMPR sites under various assay conditions, and comparison of these values with those obtained by the direct measurement of drug-induced inhibition of initial rates of nucleoside flux (representative of transport rates), and
2) detailed analysis of the physiochemical (e.g., thermodynamic) changes associated with drug interactions with [^{3}H]NBMPR sites.

As species-related differences exist in the rate of nucleoside disappearance from the circulation, valuable information concerning the relationship of the [^{3}H]NBMPR binding sites to the erythrocyte nucleoside transport systems may be obtained from a comparison of

[^{3}H]NBMPR binding site characteristics with the kinetics of nucleoside transport among erythrocytes from a variety of species.

Evidence for the interaction of several classes of CNS-active drugs with the nucleoside transport system of CNS membranes has been reviewed. However, few of the studies investigating nucleoside uptake in CNS structures (usually synaptosome preparations), and its inhibition by drugs, have measured initial rates of nucleoside flux. In fact, such studies may be technically impossible due to the rapid nature of the transport process, its lack of concentrative ability, and the fragile and often 'leaky' nature of synaptosomal preparations.

Study of the site-specific binding of the probe [^{3}H]NBMPR in CNS membranes is an alternate method by which to study the CNS nucleoside transport system. The objectives of this part of the study are:

1) to determine binding parameters of [^{3}H]NBMPR,
2) to compare the characteristics of [^{3}H]NBMPR binding sites in brain with those previously determined in human erythrocytes,
3) to determine the regional and subcellular distribution of these sites in brain, and
4) to investigate the possibility of species variations in the binding of [^{3}H]NBMPR to CNS membranes.

The relationship of [^{3}H]NBMPR binding sites to other sites, including adenosine receptors, and benzodiazepine recognition sites, can thus be investigated.

These studies provide information on the identity of CNS-located [^{3}H]NBMPR binding sites in several species, and permit an evaluation of the utility of [^{3}H]NBMPR as a probe of the nucleoside transport system, both in erythrocytes, and in CNS membranes.

II

MATERIALS AND METHODS

II. MATERIALS AND METHODS

A. Collection and Preparation of Erythrocytes

1) Collection of erythrocytes: Human blood, stored at 4°C as packed erythrocytes in citrate-phosphate-dextrose solution for 22 to 35 days, was obtained from the Blood Transfusion Service of the Canadian Red Cross Society. Fresh whole blood (5 to 10 ml) from healthy human volunteers was collected by venipuncture into heparinized (Vacutainer^R) tubes. Whole blood was also collected from rats (Sprague-Dawley, male, 150-250 g) and mice (BIOD2F, adult male) after decapitation, and from dogs (mongrels, adults of either gender) and rabbits (Dutch, male 1 to 2 kg) via a peripheral vein. All blood samples were then centrifuged at 1000 g for 10 min and the plasma and buffy coat discarded. Packed erythrocytes were stored at 4°C and used within 24 h.

2) Preparation of erythrocyte suspensions: Erythrocytes were washed three times in 20 vol. of Dulbecco's Phosphate Buffered Saline (PBS, Appendix 2) and centrifuged at 1000g for 10 min. Approximately eight drops (≈ 400 μl) of the washed, packed erythrocytes were diluted with 70 to 100 ml of PBS to obtain a final suspension containing 3 to 4 X 10^7 cells/ml. A 1:1000 dilution of this cell suspension was prepared, from which the number of cells/ml was determined with the use of a Coulter Counter (counting parameters: 1/amplification, 1;1/aperture current, 1/4 (2 mA); threshold, 10).

B. [^{3}H]NBMPR Binding Assays with Erythrocytes

1) General methodology: Assays of the site-specific binding of [^{3}H]NBMPR to erythrocytes were conducted in polypropylene microcentrifuge

tubes (1.5 ml) at 22°C. Assay mixtures were completed by the addition of cell suspension (0.5 ml) to appropriate concentrations of [^{3}H]NBMPR (in the absence or presence of potential inhibitors) to obtain 1.5 to 2.5 X 10^7 cells in a final volume of 1.0 ml. Cells were allowed to incubate with [^{3}H]NBMPR for 20 min (unless otherwise specified) to attain equilibrium. Equilibrium conditions occur when the rate of [^{3}H]NBMPR dissociation from its binding sites equals the rate of [^{3}H]NBMPR association and, therefore, the binding of a particular concentration of [^{3}H]NBMPR is maximal under these conditions. The reaction was terminated by separating the free from the cell-associated [^{3}H]NBMPR by centrifugation in an Eppendorf 5412 microcentrifuge (12000g for 10 s). The surface of the cell pellet was washed once with ice-cold PBS (1.0 ml) prior to preparation for liquid scintillation counting (Appendix 3). Nonspecific binding of [^{3}H]NBMPR was defined as NBMPR molecules which became cell-associated when the assay mixtures contained high concentrations of nitrobenzylthioguanosine (10 µM) or dipyridamole (10 µM), both of which are tightly bound inhibitors of nucleoside transport. Under these conditions, the transport inhibitors eliminated the site-specific binding of [^{3}H]NBMPR. Specific binding was defined as the total bound minus the nonspecifically bound component.

2) Individual assay procedures:

a) Determination of [^{3}H]NBMPR binding constants: [^{3}H]NBMPR binding constants, i.e., dissociation constant (K_D) and maximum number of binding sites (B_{max}), were determined by mass law analysis. Cells were incubated with a range of concentrations of [^{3}H]NBMPR (fresh cells, 0.04 nM to 1.5 nM; stored cells, 0.25 nM to 5.0 nM) and the total, nonspecific, and specific components of the binding of $[^3H]$NBMPR were determined for each $[^3H]$NBMPR concentration used in the assay.

b) Inhibition of the site-specific binding of $[^3H]$NBMPR:

(i) Determination of IC_{50} values: Each proposed inhibitor of the binding of $[^3H]$NBMPR was initially tested at a concentration of 300 μM for its ability to inhibit the site-specific binding of a K_D concentration of $[^3H]$NBMPR (fresh cells, 0.35 nM; stored cells, 1.0 nM). If the test compound, at a concentration of 300 μM, produced greater than 50% inhibition of the binding of $[^3H]$NBMPR then a range of 6 to 12 concentrations of the inhibitor was tested in order to determine a complete concentration-response curve, including the minimum effective concentration and the concentration needed for 100% inhibition. IC_{50} values were determined as the concentration of inhibitor which produced a 50% inhibition of the site-specific binding of $[^3H]$NBMPR. Triplicate determinations were made of total binding (no inhibitor present) and nonspecific binding of $[^3H]$NBMPR (usually at a K_D concentration) in each assay.

(ii) Determination of inhibition constants (K_i values): The site-specific binding of $[^3H]$NBMPR was determined for a range of $[^3H]$NBMPR concentrations (fresh cells, 0.2, 0.25, 0.33, 0.5, 0.67, 0.8 nM; stored cells, 0.5, 0.67, 0.8, 1.0, 2.0 nM) in the presence and absence of two or three concentrations of each inhibitor. The inhibitor concentrations were chosen in order to achieve approximately 30%, 50%, and 70% inhibition of the binding of a K_D concentration of $[^3H]$NBMPR. The nonspecific binding component associated with each $[^3H]$NBMPR concentration was also determined and subsequently subtracted from the total binding estimates prior to evaluation of the data by mass law analysis using a double reciprocal plot (Section F.2.d).

c) Thermodynamic and kinetic analysis of the site-specific binding of [^{3}H]NBMPR:

(i) Temperature dependence of the site-specific binding of [^{3}H]NBMPR: Binding constants for the site-specific binding of [^{3}H]-NBMPR to human erythrocytes were estimated at various temperatures, at pH 7.4. This was accomplished by incubating cells with a range of [^{3}H]NBMPR concentrations (0.04 nM to 1.5 nM) in the absence (total binding) and presence (nonspecific binding) of 10 µM dipyridamole as described in Section B.1. The incubation times used were determined previously to be sufficient for the attainment of equilibrium at each temperature (60 min at 1° to 3°C, 30 min at 10° to 15°C, 20 min at 22°C, 15 min at 28° to 30°C, and 10 min at 37°C). Data were analysed according to the van't Hoff equation (Segel, 1976) to determine changes in enthalpy (ΔH) and entropy (ΔS) associated with the binding reaction (Section F.2.g).

(ii) Temperature dependence of inhibitor interactions with the [^{3}H]NBMPR binding site: Inhibition constants (K_i) for inhibitors of the site-specific binding of [^{3}H]NBMPR were determined, as described above, at various assay temperatures. However, since the affinity of [^{3}H]NBMPR for its sites in erythrocytes varies with incubation temperature, a different range of [^{3}H]NBMPR concentrations had to be used for the determination of inhibitor K_i values at different temperatures. The concentrations of [^{3}H]NBMPR used were: 0.08, 0.1, 0.12, 0.17, 0.25, and 0.5 nM at incubation temperatures less than 25°C, and 0.15, 0.2, 0.25, 0.33, 0.5, and 0.67 nM at incubation temperatures greater than 25°C. The resulting data were analysed by the van't Hoff plot method (Segel, 1976) to determine ΔH and the ΔS values resulting from the interaction of inhibitors with the [^{3}H]NBMPR binding sites (Section F.2.g).

(iii) Dissociation rates of [^{3}H]NBMPR: Fresh human erythrocytes were incubated at 22°C with 0.35 nM [^{3}H]NBMPR for a time necessary to attain binding equilibrium (10 min). At equilibrium, the cells were sedimented by centrifugation for 10 s in an Eppendorf 5412 microcentrifuge and then resuspended in 1.0 ml of PBS, at various temperatures (4° to 37°C), containing inhibitors of the site-specific binding of [^{3}H]NBMPR. These inhibitors were present at concentrations sufficient to prevent reassociation of free [^{3}H]NBMPR (100 to 1000 times the K_i value). Assays were terminated by centrifugation (as described above) at various times after the addition of inhibitor(s) and the remaining site-bound [^{3}H]NBMPR was determined; this procedure allowed the construction of time-courses for the dissociation of [^{3}H]NBMPR from its binding sites. Dissociation rate constants (k_2) for [^{3}H]NBMPR were estimated at each temperature and for each displacing agent (Section F.2.f). These data were plotted in the form of an Arrhenius plot to determine the activation energy for the dissociation of [^{3}H]NBMPR from its sites (Section F.2.g).

C. Collection and Preparation of CNS Membranes

1) Tissue collection: Healthy mice (BIOD2F, male adult), rats (Sprague-Dawley, male, 150 to 250 g), guinea pigs (albino, female, 200 to 300 g), and rabbits (Dutch, male, 1 to 2 kg) were sacrificed by decapitation. Dogs (mongrels, adults of either gender) were anaesthetized with sodium pentobarbital prior to sacrifice. Whole brains were

removed and placed in ice-cold 0.32 M sucrose (pH 7.5).

2) Dissection techniques: Cortical tissue was separated by blunt dissection in ice-cold 0.32 M sucrose (pH 7.5) and as much white matter as possible was removed. Several guinea pig brains were further subdivided, by blunt dissection, to obtain the hippocampus, cerebellum, olfactory lobe, caudate nuclei, and lower brain stem (Glowinski & Iversen, 1966). Tissue was also obtained from the pons/medulla and the thalamus/hypothalamus regions. These tissues were washed in ice-cold 0.32 M sucrose (pH 7.5) to remove as much blood as possible, blotted dry with filter paper, and transferred to a pre-weighed , chilled, 10 ml beaker to determine tissue wet weight.

3) Preparation of P_2 membrane fraction: Membranes were prepared by a method modified from that of Gray and Whittaker (1962) and is described schematically in Figure 1. All steps involved in the procedure were performed at 4°C. Tissue was manually minced and then homogenized (5 passes at 600 rev/min in a teflon-glass homogenizer with a clearance of 0.15 to 0.23 mm) in approximately 10 vol of 0.32 M sucrose (pH 7.5). Homogenates were centrifuged at 1000g for 10 min and the supernatant was collected. The pellet (P_1) was washed twice ~~by~~ resuspension in 10 vol of 0.32 M sucrose and recentrifuged at 1000g for 10 min with collection of supernatant. These supernatants (S_1) were then pooled and centrifuged at 20000g for 30 min and the supernatant (S_2) from this centrifugation step was carefully decanted and discarded. The resulting pellet (P_2) was resuspended in the minimum possible volume of ice-cold 0.32 M sucrose (pH 7.5) with gentle vortexing. If this fraction was to be used for binding assays, it was further

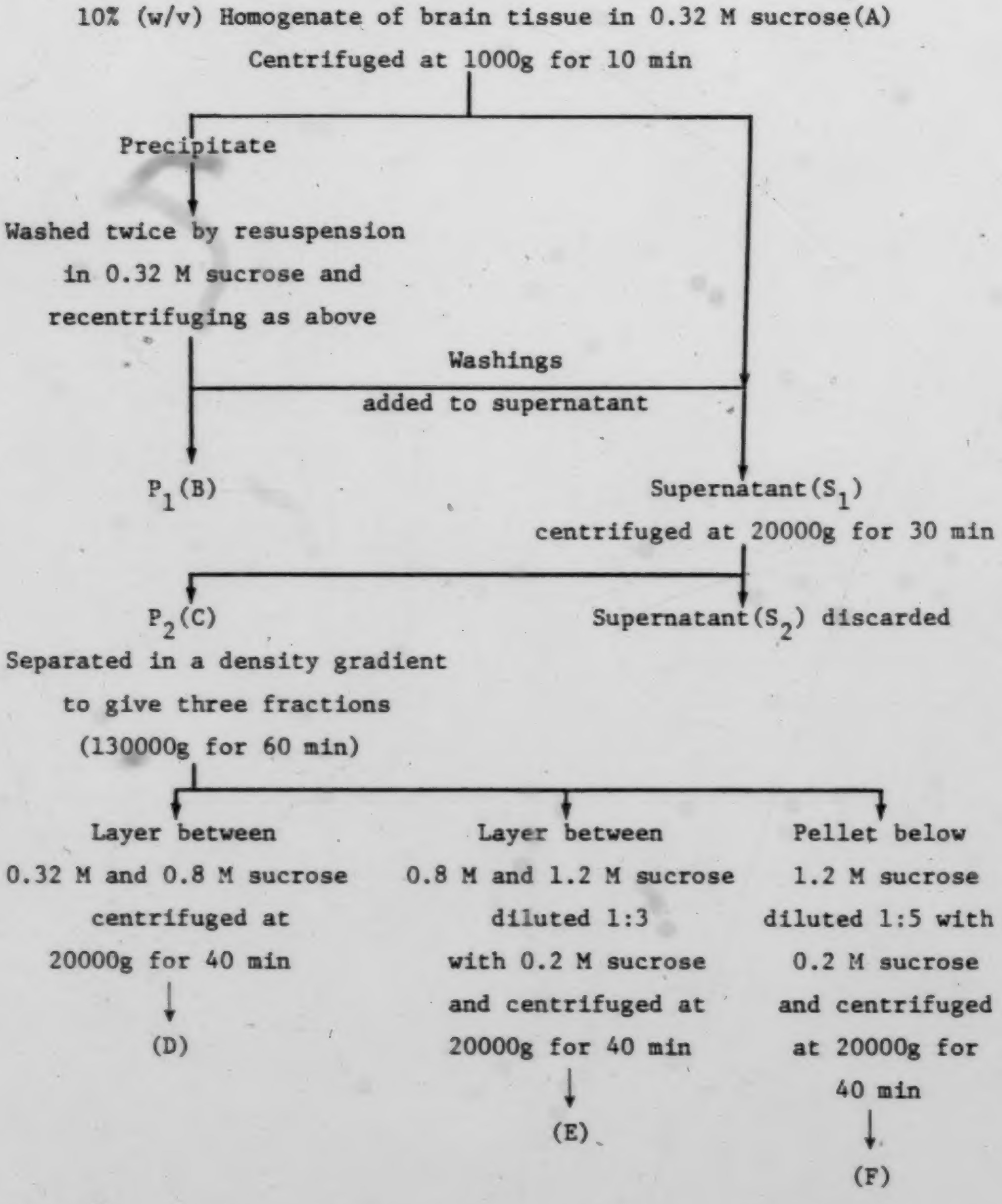

10% (w/v) Homogenate of brain tissue in 0.32 M sucrose(A)
Centrifuged at 1000g for 10 min
Precipitate
Washed twice by resuspension in 0.32 M sucrose and recentrifuging as above
Washings added to supernatant
P_1(B)
Supernatant(S_1) centrifuged at 20000g for 30 min
P_2(C)
Separated in a density gradient to give three fractions (130000g for 60 min)
Supernatant(S_2) discarded
Layer between 0.32 M and 0.8 M sucrose centrifuged at 20000g for 40 min
(D)
Layer between 0.8 M and 1.2 M sucrose diluted 1:3 with 0.2 M sucrose and centrifuged at 20000g for 40 min
(E)
Pellet below 1.2 M sucrose diluted 1:5 with 0.2 M sucrose and centrifuged at 20000g for 40 min
(F)

FIGURE 1 Scheme summarizing the preparation of subcellular fractions. The letters in parentheses(A, B, C, D, E, and F) refer to the samples used for the studies of the subcellular distribution of the binding of [^{3}H]NBMPR(Section D.1.c). Adapted from Gray & Whittaker(1962).

diluted with 0.32 M sucrose (pH 7.5) to obtain a 10 to 20 mg/ml protein suspension (see Appendix 4 for protein determinations) which was stored at 4°C and used within 24 h.

4) Preparation of synaptosomes: Partially purified synaptosomes were prepared from the P_2 fraction by sucrose density gradient centrifugation (Figure 1). The concentrated P_2 fraction (2.5 ml/gradient) was layered on sucrose density gradients consisting of 5 ml of 0.8 M sucrose (pH 7.5) layered over 5 ml of 1.2 M sucrose (pH 7.5) in 13 ml, thin-walled polyallomer ultracentrifuge tubes (Beckman, Palo Alto, Ca., USA). These tubes were then centrifuged at 130000g for 60 min in a swinging bucket rotor (Beckman, SW 41). This procedure resulted in the separation of the P_2 fraction into three distinct bands: myelin (0.32 /0.8 M sucrose interface), synaptosomes (0.8/1.2 M sucrose interface), and mitochondria (pellet). The material above the synaptosome band was carefully removed with a pasteur pipette and the synaptosomes were collected using a curved, large bore needle and syringe. The total volume of synaptosome suspension collected was noted and the suspension was diluted slowly, with constant mixing, with ice-cold 0.2 M sucrose (pH 7.5) to achieve a final sucrose concentration of approximately 0.4 M (note: the synaptosome fraction collected from the sucrose gradient contained approximately 1 M sucrose). This solution was centrifuged at 20000g for 40 min, the supernatant discarded, and the synaptosomal pellet gently resuspended in ice-cold 0.32 M sucrose (pH 7.5) to obtain a final suspension of 10 to 25 mg protein/ml, which was stored at 4°C and used within 24 h.

D. [^{3}H]NBMPR Binding Assays with CNS Membranes

1) Centrifugation assay:

a) General methodology: Assays of the site-specific binding of [^{3}H]NBMPR to CNS membranes were conducted in polypropylene microcentrifuge tubes (1.5 ml). Assay mixtures were completed by the addition of membrane suspension (20 to 40 µl) to 1.0 ml of the appropriate concentration of [^{3}H]NBMPR (in the presence and absence of proposed inhibitors) to obtain a final protein concentration of 0.25 to 0.50 mg/ml. The binding reaction was allowed to continue to equilibrium (20 min at 22°C) and was then terminated by centrifugation in an Eppendorf 5412 microcentrifuge (12000g for 2 min). The surface of the membrane pellet was washed once with 1.0 ml of ice-cold Krebs-Tris buffer (Appendix 2) prior to preparation for liquid scintillation counting (Appendix 3). Nonspecific binding of [^{3}H]NBMPR was defined as the [^{3}H]NBMPR which became membrane-associated when the assay mixtures contained nitrobenzylthioguanosine (10 µM) or dipyridamole (100 µM), both of which are tightly bound inhibitors of nucleoside transport. Under these conditions, the transport inhibitors inhibited all site-bound [^{3}H]NBMPR. Specific binding was defined as the total binding minus the nonspecific component.

The amount of membrane protein associated with the pellet, which was obtained upon termination of a binding assay, was estimated for each membrane preparation. A 'mock' binding assay (no [^{3}H]NBMPR) was performed in triplicate using the same volume of membrane suspension (20 to 40 µl) diluted into the same final volume (1.0 ml) of buffer that would subsequently be used in all the binding assays. This protein suspension was centrifuged as for the binding assays and the protein associated with the resulting pellet was determined (Appendix 4).

b) Characterization of the binding of [^{3}H]NBMPR to guinea pig CNS membranes:

(i) Determination of optimal assay conditions: Centrifugation procedure: Experiments were conducted in order to determine the minimum centrifugation time (at 12000g) required to pellet the maximum amount of membrane protein contained in 1.0 ml of the binding assay incubation medium. A known amount of membrane suspension (0.96 mg/ml) was centrifuged at 12000g for 1, 2, 3, or 5 min and the amount of protein associated with 0.5 ml of the resulting supernatant was determined. Approximately 80% of the protein added to the assay was pelleted within 1 min and another 5% sedimented during the next minute. Further centrifugation, up to 5 min, did not improve the protein recovery. Therefore, the optimum centrifugation time for pelleting protein from these binding assays was 2 min.

Wash procedure: The total, nonspecific, and specific binding of [^{3}H]NBMPR (1.0 nM and 5.0 nM) to guinea pig cortical membranes at 22°C were determined as described in Section D.1.a. Six different wash procedures were compared using ice-cold Krebs-Tris buffer; these included: (a) no wash, (b) one 0.5 ml wash, (c) two 0.5 ml washes, (d) one 1.0 ml wash, (e) two 1.0 ml washes, and (f) three 1.0 ml washes. In each case, the membrane pellet was disturbed as little as possible. The wash procedure which resulted in the lowest nonspecific binding without a reduction in site-specific binding of [^{3}H]NBMPR was a single 1.0 ml wash with ice-cold buffer.

(ii) Effect of varying protein concentration: The site-specific binding of [^{3}H]NBMPR (1.0 nM) to synaptosomal membranes was determined at 22°C, as described in Section D.1.a, using assay protein concentrations which ranged from 0.04 mg/ml to 0.85 mg/ml (maintaining the assay volumes at 1.0 ml).

(iii) Effect of pH: Synaptosomal membranes were incubated with an initial concentration of 0.35 nM [^{3}H]NBMPR at 22°C in Krebs-Tris buffers adjusted to various pH values (4 to 12) and site-specific binding of [^{3}H]NBMPR was estimated as described in Section D.1.a. Membrane pellets were washed with ice-cold buffer of the same pH as that used for the incubation medium.

(iv) Association rate of [^{3}H]NBMPR: Synaptosomal membranes were incubated with an initial concentration of 0.35 nM or 1.0 nM [^{3}H]NBMPR at 22°C and at a pH of 7.4 as described in Section D.1.a. At various times after the addition of protein (1 to 30 min), the assays were terminated by centrifugation and the pellet was assayed for site-bound [^{3}H]NBMPR. The association rate of [^{3}H]NBMPR (0.35 nM, initial concentration) to synaptosomal membranes was also determined at 4°C using a 1 to 60 min time-course.

(v) Effect of temperature: Binding constants for the site-specific binding of [^{3}H]NBMPR to guinea pig synaptosomal membranes were estimated at various incubation temperatures. This was accomplished by incubating membranes with a range of concentrations of [^{3}H]NBMPR (0.04 nM to 1.5 nM) in the absence (total binding) and presence (nonspecific binding) of 100 μM dipyridamole as described in Section D.1.a. The incubation times used were determined previously to be sufficient for the attainment of equilibrium at each temperature

(20 min at 55°C, 37°C, and 22°C; 60 min at 4°C). All assay wash procedures were conducted using ice-cold buffer (pH 7.4).

(vi) Effect of ions: An estimation of the ion dependence of the binding of [^{3}H]NBMPR to guinea pig synaptosomal membranes was determined by comparing [^{3}H]NBMPR binding constants derived from assays conducted at 22°C in: (a) Krebs-Tris buffer, prepared as described in Appendix 2, (b) 15 mM Tris + 155 mM NaCl + 10 mM Na2EDTA, (c) 50 mM Tris + 10 mM Na2EDTA, or (d) 50 mM Tris only. All buffers were adjusted to pH 7.4 prior to use. The wash procedure was performed with the same buffer, at 0°C to 4°C, as that used for the incubation medium.

(vii) Effect of treatment of membranes with adenosine deaminase (EC 3.5.4.4): Synaptosomal membranes were preincubated at 4°C with adenosine deaminase (9 X 10^{-2} units/ml, final concentration) for at least 12 h in order to metabolize endogenous adenosine in the membrane preparation. Binding constants for [^{3}H]NBMPR in these membranes were then determined and compared to those determined simultaneously in untreated membranes.

c) Subcellular distribution of the binding of [^{3}H]NBMPR: Cortical tissue was homogenized and fractionated as described in Section C. Measured aliquots of membrane suspension from each step in the fractionation procedure (Figure 1) were removed and prepared as described below: Sample A - crude homogenate; Sample B - washed P_1 pellet resuspended in 0.32 M sucrose; Sample C - P_2 membrane suspension; Sample D - material at the 0.32/0.8 M sucrose interface of the sucrose gradient, removed with a pasteur pipette; Sample E - synaptosomes removed from the 0.8/1.2 M sucrose interface of the sucrose gradient, prepared as described in Section C.4; Sample F - pelleted material from the sucrose gradient, resuspended and diluted 1:5 with ice-cold 0.2 M sucrose (pH 7.5). Each of the samples (A - F) were centrifuged at 20000g for 40 min, the supernatant removed, and the pellets resuspended in 2 ml of ice-cold 0.32 M sucrose (pH 7.5). A sufficient volume of membrane suspension (20 to 40 μl) was added to the binding assay (final vol 1.0 ml) to achieve approximately 0.2 to 0.5 mg protein/assay. [^{3}H]NBMPR binding constants were determined, for each membrane fraction (A - F), using concentrations of [^{3}H]NBMPR ranging from 0.04 nM to 1.5 nM.

d) Regional distribution of the binding of [^{3}H]NBMPR: P_2 membranes were prepared (Section C.3) from each of the following brain regions: cortex, cerebellum, olfactory lobe, hippocampus, caudate nuclei, thalamus/hypothalamus, pons/medulla, and lower brain stem. A sufficient volume of each membrane suspension (20 to 40 μl) was added to the binding assays to achieve 0.2 to 0.5 mg protein/assay (1.0 ml). [^{3}H]NBMPR binding constants were determined, in each brain region, as described previously (Section D.1.a) using concentrations of [^{3}H]NBMPR ranging from 0.04 nM to 1.5 nM. Inhibition constants (K_i values) were determined for selected inhibitors of the site-specific binding of [^{3}H]NBMPR (see below) to membranes prepared from regions of high and low [^{3}H]NBMPR binding site density.

e) Inhibition of the binding of [^{3}H]NBMPR: Inhibitors were evaluated in the same manner as that described for erythrocytes (Section B.2.b). Concentration-inhibition curves (percent inhibition of the site-specific binding of [^{3}H]NBMPR against inhibitor concentration) were obtained using 0.35 nM [^{3}H]NBMPR and K_i values were ascertained by double reciprocal plot analysis using initial concentrations of [^{3}H]NBMPR ranging from 0.2 nM to 0.8 nM.

f) Species differences in the binding of [^{3}H]NBMPR to CNS membranes: P_2 membranes were prepared from cortical tissue of each species as described previously for guinea pig cortical tissue (Section C.3). Binding constants for the interaction of [^{3}H]NBMPR with its binding sites in each species were determined as described above and in Section F.2.a. The range of concentrations of [^{3}H]NBMPR used in these assays varied depending on the source of the tissue: guinea pig, rat, and mouse, 0.04 to 1.5 nM; rabbit, 0.08 to 5.0 nM; dog, 0.25 to 15.0 nM. The ability of dipyridamole and diazepam to inhibit the specific binding of [^{3}H]NBMPR to membranes derived from each species was determined as follows. A range of 10 to 16 concentrations of these inhibitors was tested against the binding of a K_D concentration of [^{3}H]NBMPR (rat, 0.15 nM; mouse, 0.15 nM; guinea pig, 0.35 nM; dog, 4.0 nM). If cortical membranes from a particular species appeared to display more than one binding site for [^{3}H]NBMPR (curvilinear Scatchard plots) then the above experiment was performed using both the 'average' K_D concentration of [^{3}H]NBMPR (see Section F.2.a.ii) and a concentration of [^{3}H]NBMPR which was 5 to 12 fold lower than the 'average' K_D concentration. In rat CNS membranes, K_i values were determined for dipyridamole and several benzodiazepines using initial assay concentrations of [^{3}H]NBMPR of 0.2, 0.25, 0.33, 0.5, 0.67, and 0.8 nM.

2) Filtration assay:

a) General methodology: A concentrated cortical membrane (P_2 or synaptosomal) suspension was prepared as described in Section C, and diluted with ice-cold 0.32 M sucrose (pH 7.5) to obtain a final protein concentration of 8 mg/ml to 12 mg/ml. The exact protein concentration of a 1:80 dilution (in 50 mM Tris buffer) of this membrane suspension was determined as described in Appendix 4, using 50 mM Tris (pH 7.4) as the buffer. Immediately prior to the binding assay, the concentrated cortical membrane suspension in 0.32 M sucrose was diluted 1:12.5 with ice-cold buffer (50 mM Tris, pH 7.4) to obtain a protein concentration of 0.64 mg/ml to 0.96 mg/ml, and was then kept on ice.

Filtration assays of the site-specific binding of [^{3}H]NBMPR to cortical membranes were conducted, at 22°C, in 5 ml sample vials using 50 mM Tris buffer (pH 7.4). Assay mixtures were completed by the addition of 0.5 ml of membrane suspension to 0.5 ml of the appropriate concentration of [^{3}H]NBMPR (in the presence and absence of inhibitors) to obtain a final protein concentration of 0.32 mg/ml to 0.48 mg/ml in the incubation mixture. The binding reaction was allowed to continue to equilibrium (20 min at 22°C) and then rapidly terminated by pouring the incubation mixture through Whatman GF/B (2.4 cm) glass fibre filters (presoaked with 50 mM Tris), under vacuum. The incubation vials, and subsequently the filters, were rinsed twice with 4 ml aliquots of ice-cold 50 mM Tris buffer and the washed filters were then transferred to the appropriate liquid scintillation vials. Nonspecific binding was defined as the amount of membrane-associated [^{3}H]NBMPR when binding assays were conducted in the presence of 100 µM dipyridamole. The filtration apparatus was washed thoroughly between with distilled water.

b) Comparison of the binding of $[^3H]$NBMPR to intact synaptosomes with its binding to lysed synaptosomes: Binding constants for the site-specific binding of $[^3H]$NBMPR to intact and lysed synaptosomes were determined by incubating the membranes with a range of concentrations of $[^3H]$NBMPR (0.04 nM to 1.5 nM). Intact synaptosomes were prepared as described in Section C.4. Lysed synaptosomes were prepared by diluting concentrated synaptosomal suspension (8 to 12 mg/ml protein in 0.32 M sucrose) 1:12.5 with ice-cold water ($pH \simeq 7.4$) and mixing vigorously. The binding assays were then conducted in the same manner as those utilizing intact synaptosomes (Section D.2.a) with the exception that 5 mM Tris buffer (pH 7.4) was used in the lysed synaptosome assays.

c) Inactivation of $[^3H]$NBMPR binding sites:

(i) Trypsin inactivation: Concentrated synaptosomal membrane suspension (8 to 12 mg/ml protein in 0.32 M sucrose) was diluted 1:12.5 with a trypsin solution (2 units/ml) prepared in 50 mM Tris buffer (pH 7.4). Synaptosomal membranes were incubated with trypsin for a least 16 h at 4°C prior to determination of the $[^3H]$NBMPR binding constants using concentrations of $[^3H]$NBMPR of 0.04 nM to 1.5 nM.

(ii) Thermal inactivation: Synaptosomal membrane suspension was heated to 60°C for 1 h, then cooled to 4°C prior to the determination of the $[^3H]$NBMPR binding constants using concentrations of $[^3H]$NBMPR of 0.04 nM to 1.5 nM.

d) Determination of the dissociation rate of $[^3H]$NBMPR from its sites in cortical membranes: P_2 membranes were prepared from cortical tissue as described in Section C.3. Binding assays were initiated by the addition of 40 µl of concentrated membrane suspension (8 to 12 mg/ml protein in 0.32 M sucrose) to 1.0 ml of incubation medium to obtain a final protein concentration of 0.32 mg/ml to 0.48 mg/ml in the incubation mixture. The incubation medium contained $[^3H]$NBMPR, at an initial concentration of approximately 3 times the K_D concentration (rat, 0.35 nM; rabbit, 2.0 nM; guinea pig, 0.8 nM) in 50 mM Tris buffer (22°C, pH 7.4). Dipyridamole (100 µM) was included in three assay mixtures in order to estimate the nonspecific binding component for experiments with each species. Incubations were allowed to continue until equilibrium was attained. At this time, a known inhibitor (40 µl to 100 µl) of the binding of $[^3H]$NBMPR was added to the incubation medium to attain a final concentration sufficient to inhibit all site-bound $[^3H]$NBMPR (>100 times its K_i concentration for the inhibition of the site-specific binding of $[^3H]$NBMPR). Assays were then terminated by filtration (as described in Section D.2.a) at various times after the addition of inhibitor, and the amount of site-bound $[^3H]$NBMPR remaining was determined; this procedure allowed the construction of time-courses for the dissociation of $[^3H]$NBMPR from its binding sites.

E. Adenosine Deaminase Assays

The rate of conversion of adenosine to inosine by adenosine deaminase (from calf intestinal mucosa)(EC 3.5.4.4) was determined spectrophotometrically at 22°C by recording the decrease in absorbance at 260 nm (see Appendix 5 for spectral data) over a period of 3 min using a Gilford spectrophotometer and chart recorder. Assays were conducted in u.v. absorption cells and incubations were initiated by the addition of 25 µl of adenosine deaminase (3×10^{-2} units) to 2 ml of incubation medium containing adenosine (45 µM) in the presence and absence of the appropriate concentration of potential adenosine deaminase inhibitors. Several benzodiazepines (at 100 µM), dipyridamole (at 100 µM), hydroxynitrobenzylthioguanosine (at 12 µM), and the solvent, dimethylsulfoxide (1.25% v/v in PBS), were compared with deoxycoformycin, a recognized adenosine deaminase inhibitor, for their ability to inhibit the activity of adenosine deaminase.

F. Data Analysis

1) Computer analysis: Data on punched tape, obtained from a teletype printer of a liquid scintillation counting system, was entered into a computer (Amdahl 470 V/8, The University of Alberta), via a teletype tape reader, using a 'READTAPE' program and was then analysed by one of two data analysis programs: 'JIM1' or 'JIM2' (Appendix 6). JIM1 provided only quench correction. JIM2 was used to calculate, (a) site-specific binding of [^{3}H]NBMPR at various inhibitor concentrations, and (b) [^{3}H]NBMPR dissociation rates. It provided background and quench correction, and conversion of dpm to either, (a) molecules

of [^{3}H]NBMPR bound per cell, or (b) fmol of [^{3}H]NBMPR bound per mg protein. Also, it related the amount of [^{3}H]NBMPR bound in each sample to a 100% control sample (no inhibitor present) and expressed this information as % binding of [^{3}H]NBMPR at each inhibitor concentration (for inhibition studies) or at each time after the addition of inhibitor (for dissociation rate studies).

2) Graphical analysis:

a) Determination of the binding parameters of [^{3}H]NBMPR: Data obtained from studies of the binding of a range of concentrations of [^{3}H]NBMPR to cells or membranes were analysed by two procedures:
(i) The amounts of cell- or membrane-associated [^{3}H]NBMPR (total, nonspecific, and specific components) were plotted against the final concentrations of [^{3}H]NBMPR present in the assay medium (determined as the initial concentration of [^{3}H]NBMPR minus the concentration bound to the cells or membranes) in order to examine the concentration dependence of the total, specific, and nonspecific components.
(ii) Site-specific binding was also analysed by mass law analysis (Scatchard plot) by plotting the ratio of site-bound [^{3}H]NBMPR to final, free [^{3}H]NBMPR concentrations (B/F) against the site-bound [^{3}H]NBMPR (B). A line was fitted to the data by linear regression (least squares method) from which the maximum number of binding sites (B_{max}) could be determined from the intercept on the abscissa, and the dissociation constant (K_D) could be determined as the negative reciprocal of the slope. For biphasic Scatchard plots, the data were separated into two independent linear functions by subtracting the lower affinity component from the curvilinear function (Figure 2). The K_D for each component and the respective B_{max} values were then estimated

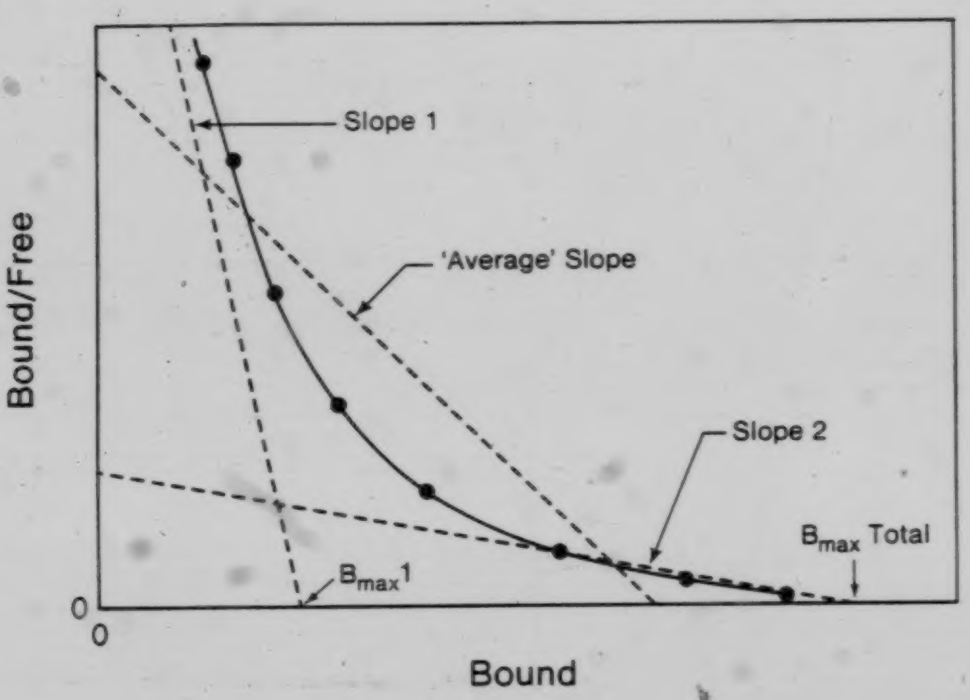


FIGURE 2 Analysis of biphasic Scatchard plots.

a) The 'average' slope was calculated by simple linear regression analysis using all experimental data points.

b) Slope 2 was fitted as the tangent to the 'low-affinity' portion of the curve, as shown.

c) Slope 1 was determined by subtracting the line of Slope 2 from the curvilinear function.

d) B_{max} 'total' and B_{max} 1(maximum number of 'high-affinity' sites) were determined as the abcissa intercepts of Slope 2 and Slope 1, respectively.

e) B_{max} 2(maximum number of 'low-affinity' sites) could then be calculated as B_{max} 'total' - B_{max} 1.

as shown in Figure 2.

b) Determination of IC_{50} values: IC_{50} values (concentration of inhibitor required to inhibit site-specific binding of $[^3H]$NBMPR by 50%) of various compounds were determined from plots of site-specific binding of $[^3H]$NBMPR against a range of inhibitor concentrations by fitting simple linear regression lines to data between 20% and 80% inhibition of the control binding of $[^3H]$NBMPR. Biphasic inhibition plots were analysed as two separate inhibition curves, with IC_{50} values for each phase determined directly from the graph, as outlined in Figure 3.

c) Determination of Hill coefficients: Hill coefficients (n_H) were calculated for the site-specific binding of $[^3H]$NBMPR as the slopes of simple linear regression lines fitted to plots of $\log[B/(B_{max} - B)]$ against the concentration of $[^3H]$NBMPR, where B = site-bound $[^3H]$NBMPR at a particular concentration of $[^3H]$NBMPR, and B_{max} = the maximum number of binding sites for $[^3H]$NBMPR. Similarly, Hill coefficients were calculated for inhibitors of the binding of $[^3H]$NBMPR from plots of $\log[B/(B_{max} - B)]$ against inhibitor concentration, where B, in this case, equals the site-bound $[^3H]$NBMPR at a particular concentration of inhibitor.

d) Determination of K_i values: Inhibition constants (K_i values) and the nature of the inhibition (i.e., competitive, noncompetitive, or uncompetitive) for inhibitors of the binding of $[^3H]$NBMPR were determined using double reciprocal plot analysis. The reciprocals of the site-specific binding of $[^3H]$NBMPR (1/B), in the presence and absence of 2 or 3 concentrations of each inhibitor, were plotted against the reciprocals of the final concentrations of $[^3H]$NBMPR (1/F).

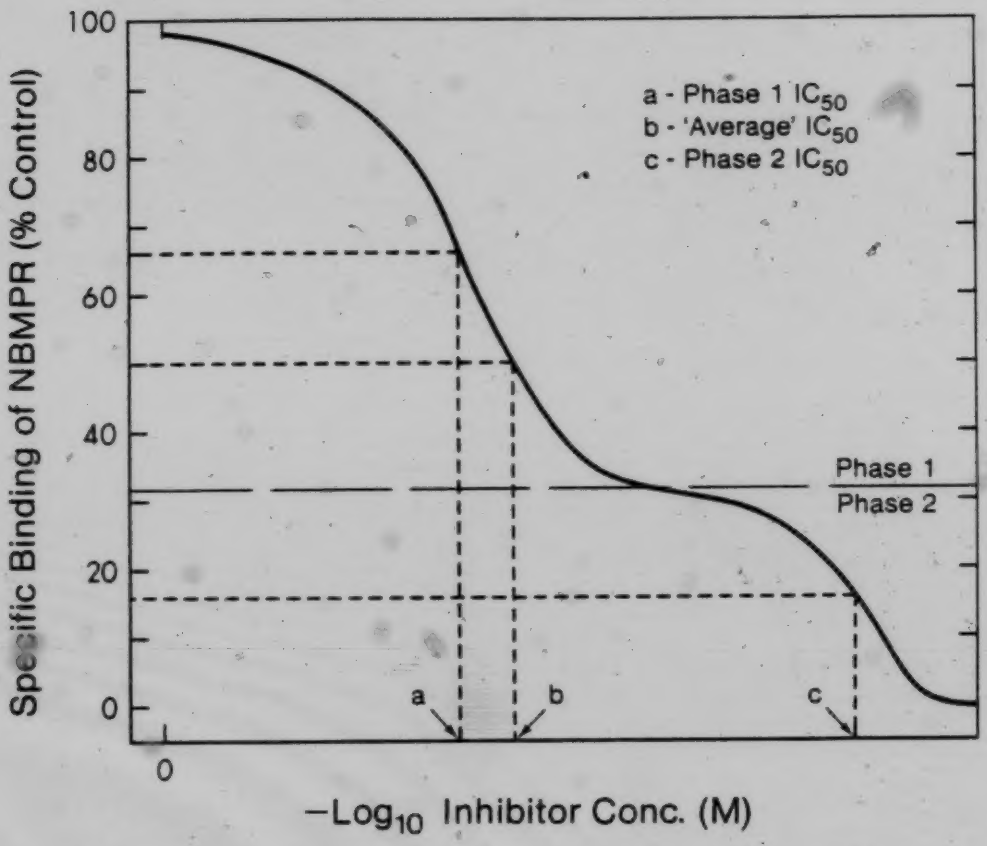


FIGURE 3 Analysis of biphasic concentration-inhibition plots. IC_{50} values for each phase were determined directly from the graph as indicated.

Slopes of these plots were determined and K_i values were calculated for each inhibitor concentration according to the following equation:

$$K_i = \left[\frac{\text{slope with inhibitor}}{\text{slope without inhibitor (control)}} - 1\right] \cdot \text{[inhibitor conc]}$$

which is derived from the Michaelis Menten equation (Lehninger, 1975).

e) Determination of association rate constants: Observed association rate constants (k_{ob}) of the binding of [^{3}H]NBMPR to its sites were calculated as the slopes of simple linear regression lines fitted to data plotted as $\ln[B_e/(B_e - B)]$ against association time, where B = site-bound [^{3}H]NBMPR at a time after initiation of the binding reaction, and B_e = the amount of site-bound [^{3}H]NBMPR at equilibrium.

f) Determination of dissociation rate constants: Rate constants for dissociation (k_2) of [^{3}H]NBMPR from its binding sites were calculated as the negative slopes of simple linear regression lines fitted to data plotted as $\ln[B/B_o]$ against dissociation time, where B = site-bound [^{3}H]NBMPR at various times, and B_o = site-bound [^{3}H]NBMPR at time zero. Biphasic dissociation plots were resolved into linear components in the same manner as that described for biphasic Scatchard plots (Section F.2.a), and k_2 values were calculated for each component.

g) Determination of thermodynamic constants: ΔH and ΔS values associated with [^{3}H]NBMPR and inhibitor interactions with the NBMPR site were determined from plots of $\ln K_A$ against $1/T$, where K_A is the association constant ($1/K_D$, or $1/K_i$ for inhibitors) of a compound for the [^{3}H]NBMPR binding site, and T is the temperature (°K). The equation of the linear function (fitted by simple linear regression) is $\ln K_A = \Delta H/RT + \Delta S/R$, where R is the gas constant (8.314 $J°K^{-1}$); this is known as the van't Hoff equation (Segel, 1976). Gibbs free energy changes (ΔG) were calculated according to the equation $\Delta G = -RT \ln K_A$. Independent estimates of ΔS could then be obtained at each incubation temperature from the relationship $T\Delta S = \Delta H - \Delta G$.

Dissociation rate constants (k_2) for [^{3}H]NBMPR were calculated at different incubation temperatures as described in Section F.2.f. A plot of ln k_2 against 1/T°K (Arrhenius plot) resulted in a linear function, the slope of which equals $E_a(-)/R$, where R is the gas constant, and $E_a(-)$ is the activation energy for the dissociation of [^{3}H]NBMPR from the binding sites.

All data are reported as mean ± s.e.mean which is sometimes followed by a number in parentheses that represents the number of replicate determinations.

G. Chemicals and Drugs

[G-^{3}H]Nitrobenzylthioinosine (sp. act. of 16 Ci/mmol) was purchased from Moravek Biochemicals Inc., and repurified (by Dr. A.R.P. Paterson, Cancer Research Unit, University of Alberta), if necessary, to greater than 98% radiochemical purity by high performance liquid chromatography using a C_{18} μBondapak column (Waters) eluted with methanol-water solutions. Nitrobenzylthioinosine, nitrobenzylthioinosine phosphate, nitrobenzylthioguanosine, hydroxynitrobenzylthioguanosine, guanosine, cytidine, and thymidine were kindly provided by Dr. A.R.P. Paterson, Cancer Research Unit (McEachern Laboratory), University of Alberta. Lorazepam and oxazepam were donated by Wyeth Ltd., and all other benzodiazepines were supplied by Hoffmann-La Roche

Ltd. β-Carbolines and phenothiazines were donated by Dr. G. Baker, Neurochemical Research Unit, University of Alberta. Morphine sulphate was obtained from Dr. G. Frank, Department of Pharmacology, University of Alberta. Other compounds, donated by their manufacturers were, dipyridamole (Boehringer Ingelheim Ltd.), hexobendine (Chemie Linz AG), dilazep (Hoffmann-La Roche Ltd.), lidoflazine (Janssen Pharmaceutica), and papaverine (Eli Lilly & Co.). All other reagents, chemicals, and enzymes used were purchased from their respective suppliers.

Preparation of drug solutions: Most drug solutions were prepared on the day of use with the buffer which was appropriate for each binding assay. The exact concentration of all solutions of nucleosides and nucleoside derivatives were determined spectrophotometrically (Appendix 5). Drugs which were not soluble in buffer were prepared in dimethylsulfoxide (DMSO), and diluted with buffer so that less than 3% DMSO was present in assay media. The 6-thiopurine nucleoside derivatives (with the exception of nitrobenzylthioinosine phosphate which is readily soluble in buffer) were prepared in either DMSO, just prior to use, or buffer, with stirring for 12 h at 22°C. A [^{3}H]NBMPR stock solution ($\simeq$ 31 μM) was prepared in 50% methanol, and stored at -20°C with further dilutions in buffer (22°C) being performed only as necessary.

III

RESULTS

III. RESULTS

A. Binding of $[^3H]$NBMPR to Human Erythrocytes

1) $[^3H]$NBMPR binding constants: The $[^3H]$NBMPR, which was associated at equilibrium with fresh and stored human erythrocytes, consisted of two components, one of which was site-specific and saturable; the cell content of the other $[^3H]$NBMPR component (nonspecific) was proportional to free $[^3H]$NBMPR concentration remaining in the assay medium and constituted less than 10% of the total cell-associated $[^3H]$NBMPR at the concentrations used (Figure 4). Mass law analysis of site-specific binding data (Figure 5A) indicated the existence of a single class of sites for $[^3H]$NBMPR in erythrocytes from both fresh and stored blood. These sites displayed no apparent binding cooperativity as indicated by Hill coefficients which were not different from unity (Figure 5B, Table 1). Observed densities of $[^3H]$NBMPR binding sites on erythrocytes from fresh and stored blood were similar (Figure 5A, Table 1). However, the apparent affinity for $[^3H]$NBMPR of the binding sites on erythrocytes from fresh blood was approximately three-fold higher than that of the sites on stored cells (Table 1).

2) Inhibition of the binding of $[^3H]$NBMPR: Several recognized inhibitors of nucleoside transport were potent and apparently competitive inhibitors of the site-specific binding of $[^3H]$NBMPR to human erythrocytes. The affinity of each of these compounds for the $[^3H]$NBMPR site of stored erythrocytes was similar to their affinity for the $[^3H]$NBMPR site in fresh cells (Table 2); dilazep was the most potent inhibitor with a K_i value of 0.29 nM in fresh erythrocytes (Figure 6) followed by, in decreasing order of affinity, hexobendine, dipyridamole, lidoflazine,

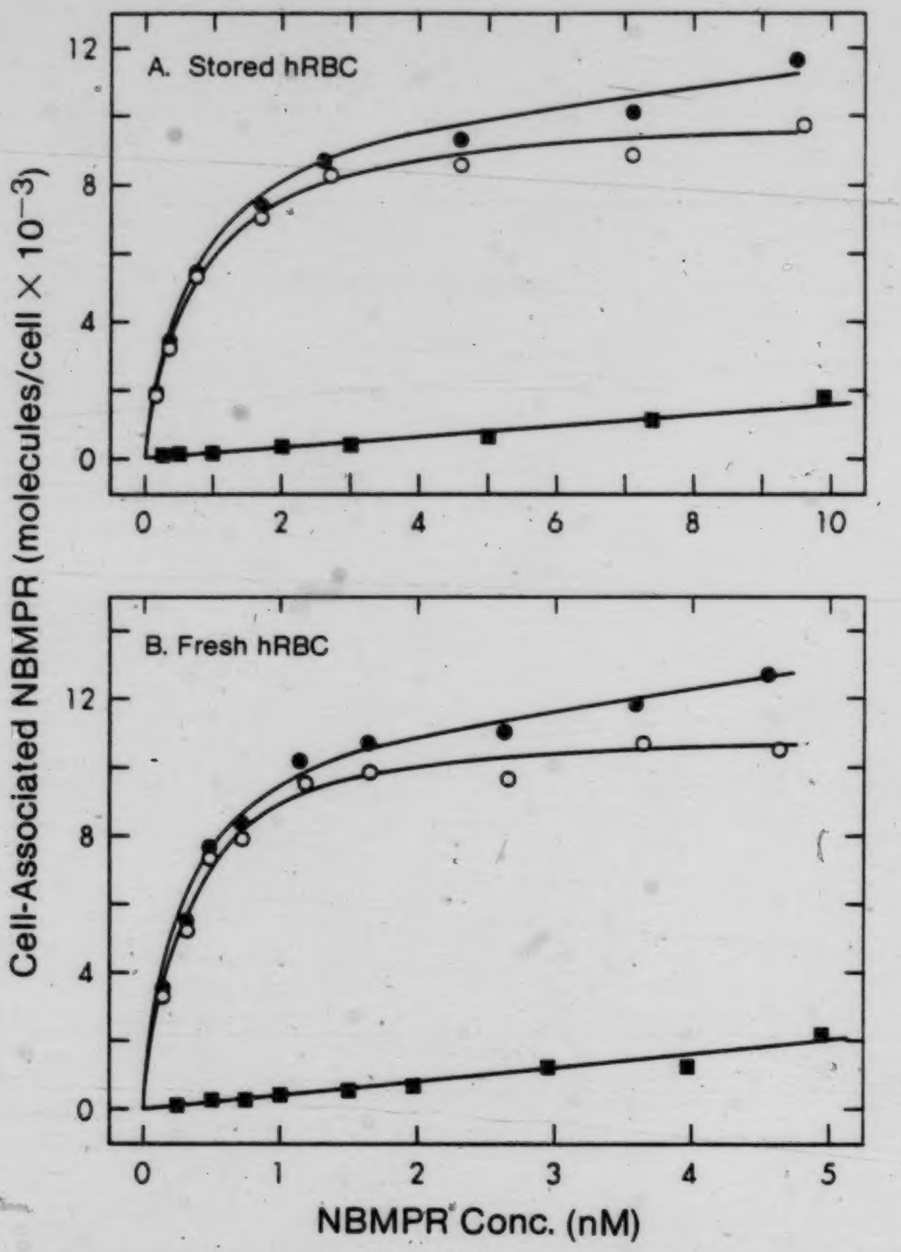


FIGURE 4 Concentration dependence of the binding of [^{3}H]NBMPR to stored(A) and fresh(B) human erythrocytes. These are representative plots which are not different from those obtained from 6 other experiments. Total binding(●); nonspecific binding(■); specific binding(○).

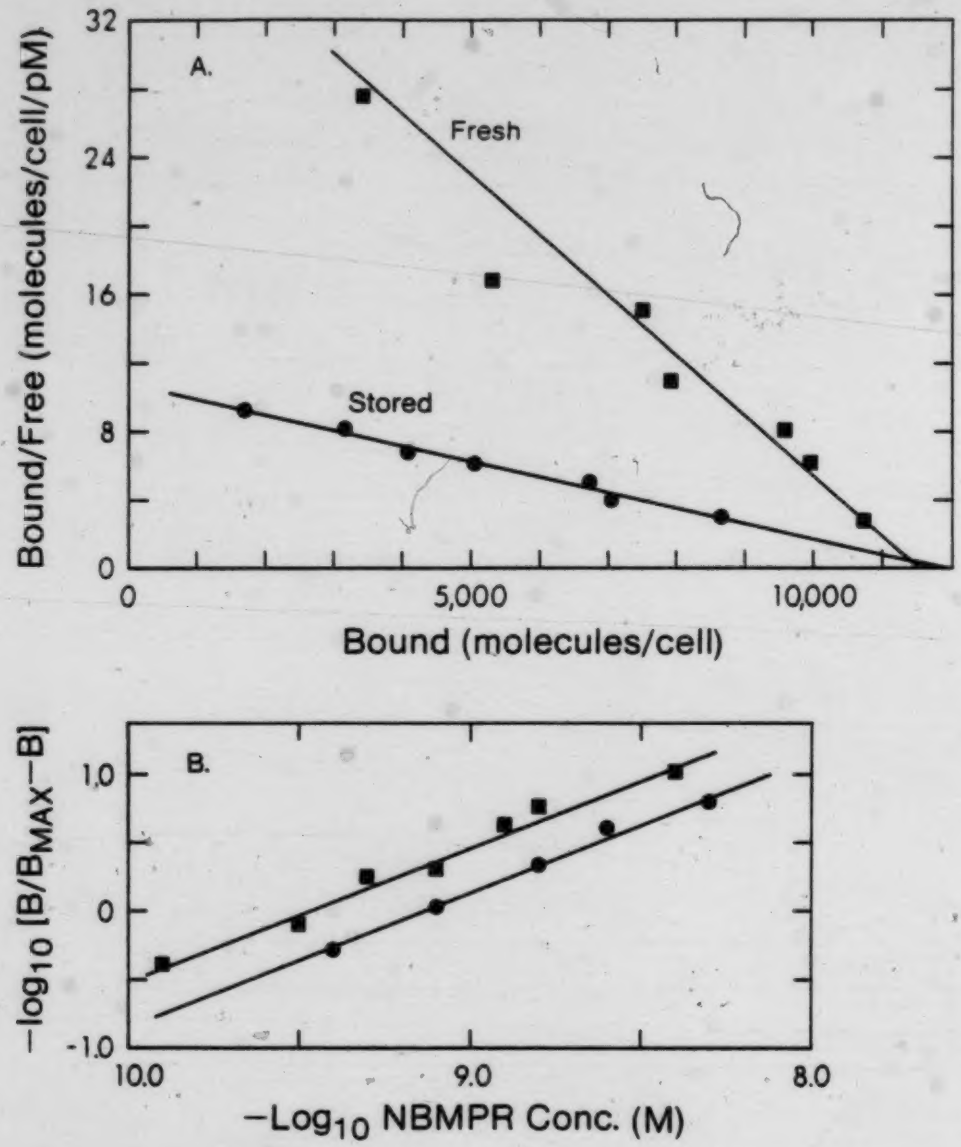


FIGURE 5 Scatchard(A) and Hill(B) plots of the site-specific binding of [^{3}H]NBMPR to human erythrocytes. These plots were obtained using data for fresh(■) and stored(●) human erythrocytes which is shown in Figure 4.

TABLE 1 Comparison of the binding parameters of [^{3}H]NBMPR in fresh and stored human erythrocytes at 22°C.

	B_{max} (molecules/cell)	K_D (nM)	n_H
Stored hRBC	12000 ± 1100(6)	0.97 ± 0.13(6)	1.02 ± 0.02(6)
Fresh hRBC	11000 ± 600(9)	0.31 ± 0.02(9)	0.98 ± 0.01(3)

Results are the means ± s.e.mean from the number of experiments indicated in parentheses.

TABLE 2 Comparison of the inhibition constants(K_i) of various agents for the inhibition of the site-specific binding of $[^3H]$NBMPR to both fresh and stored human erythrocytes.

Inhibitor	Fresh hRBC	Stored hRBC
	K_i (nM)	K_i (nM)
Dilazep	0.29 ± 0.05 (8)	0.85 ± 0.12 (6)
Hexobendine	1.9 ± 0.2 (6)	1.8 ± 0.1 (12)
Dipyridamole	1.9 ± 0.4 (6)	4.5 ± 0.2 (9)
Lidoflazine	42 ± 6 (6)	35 ± 4 (6)
Papaverine	3500 ± 600 (6)	4200 ± 1500 (6)
	K_i (μM)	K_i (μM)
Ro 5-4864	2.2 ± 0.6 (8)	-
Diazepam	6.8 ± 1.1 (7)	9.8 ± 1.7 (6)
Clonazepam	24 ± 5 (6)	51 ± 5 (6)*
Chlordiazepoxide	-	59 ± 5 (6)
Lorazepam	45 ± 7 (7)	84 ± 9 (6)

* This K_i value was calculated from the corresponding IC_{50} value according to the equation $K_i = IC_{50}/[1 + (\text{NBMPR conc}/K_D)]$ where 'NBMPR conc' is the concentration of $[^3H]$NBMPR used for the IC_{50} determination and 'K_D' is the dissociation constant of $[^3H]$NBMPR. All other K_i values were determined directly by double reciprocal plot analysis.

Results are the means ± s.e.mean from the number of experiments indicated in parentheses.

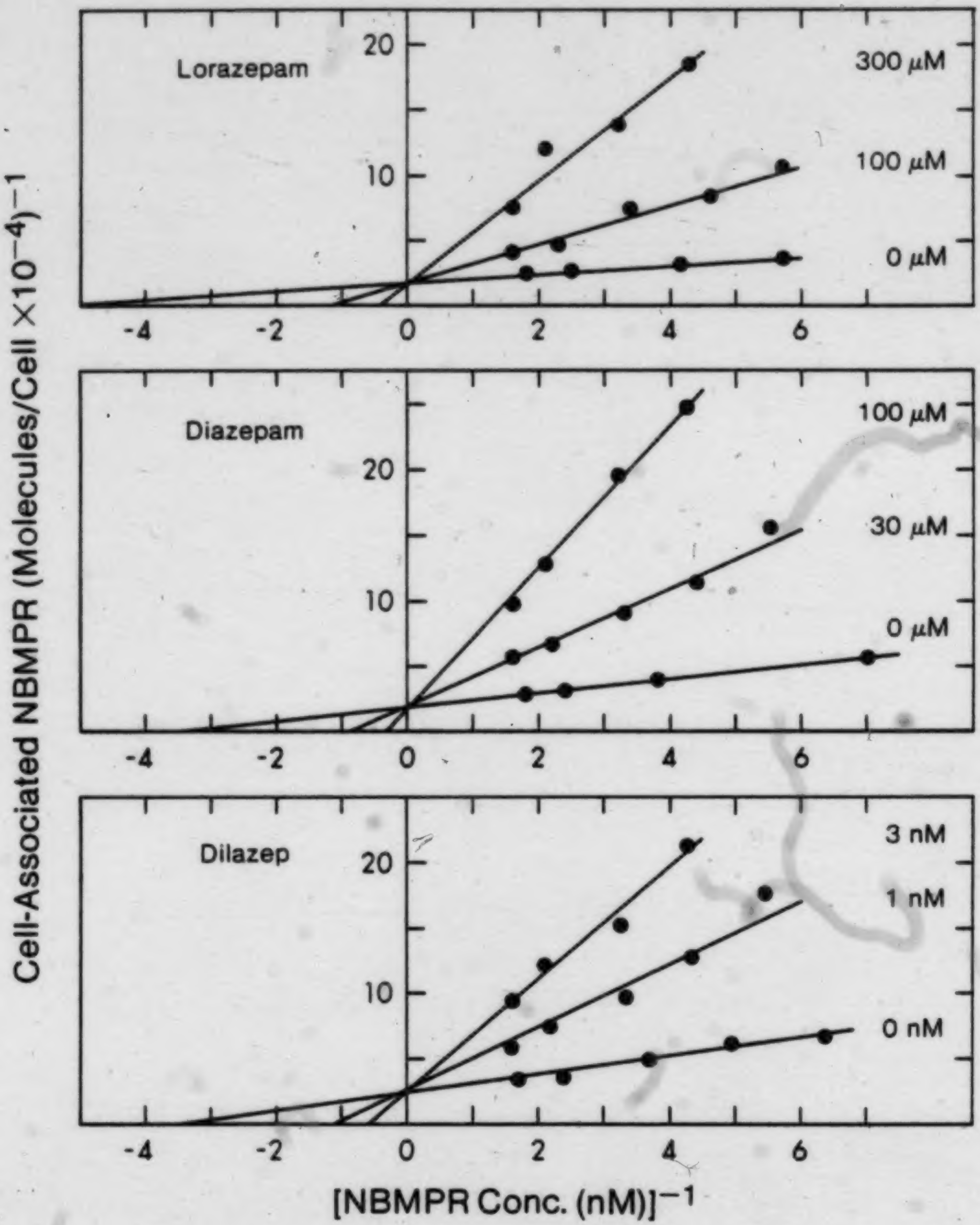


FIGURE 6 Double reciprocal plot analysis of the inhibition of the site-specific binding of $[^3H]$NBMPR to fresh human erythrocytes by lorazepam, diazepam and dilazep.

and papaverine (Table 2). Nitrobenzylthioguanosine and hydroxynitrobenzylthioguanosine, cogeners of NBMPR and themselves potent inhibitors of nucleoside transport, were also very effective inhibitors of the binding of [^{3}H]NBMPR to human erythrocytes (Table 3). Each of the above mentioned compounds inhibited the site-specific binding of [^{3}H]NBMPR in a concentration-dependent manner (Figure 7A) and, with the exception of lidoflazine and papaverine (n_H values of 0.70 and 0.48, respectively), each of these inhibitors exhibited Hill coefficients for the inhibition of the binding of [^{3}H]NBMPR which were not different from unity (Table 3). Due to large errors associated with the experimental determination of Hill coefficients, only those values which were less than 0.75 were considered to be different from unity; Hill coefficients which are not different from unity indicate the absence of cooperative site interactions.

Benzodiazepines also inhibited the site-specific binding of [^{3}H]NBMPR in a concentration-dependent manner (Figure 7B). However, the affinities of the benzodiazepines for the [^{3}H]NBMPR site in human erythrocytes were generally more than 100-fold lower than the affinities of the nucleoside transport inhibitors, dipyridamole and dilazep (Table 2 & 3). None of the benzodiazepines tested inhibited the binding of [^{3}H]NBMPR at concentrations less than 1 μM (Figure 7B). Flunitrazepam, nitrazepam, diazepam, desmethyldiazepam, the water soluble benzodiazepine, midazolam, and the 'non-neuronal' benzodiazepine site ligand, Ro 5-4864, were the most potent benzodiazepines at the [^{3}H]NBMPR site, followed by clonazepam, oxazepam and chlordiazepoxide (Table 3 & 4). Lorazepam, which is 5 times as effective an anxiolytic as diazepam, was about 6-fold weaker than diazepam as an inhibitor of the binding of

TABLE 3 Inhibition of the site-specific binding of [^{3}H]NBMPR to stored human erythrocytes by various recognized nucleoside transport inhibitors and benzodiazepines.

Inhibitor	IC_{50}*	n_H
Nucleoside Transport Inhibitors	(nM)	
Dilazep	2.0 ± 0.2	0.90 ± 0.04
Nitrobenzylthioguanosine	2.5 ± 0.2	0.97 ± 0.02
Hexobendine	3.4 ± 0.6	0.75 ± 0.08
Dipyridamole	10 ± 1	0.93 ± 0.05
Hydroxynitrobenzylthioguanosine	23 ± 1	0.89 ± 0.04
Lidoflazine	72 ± 10	0.70 ± 0.01
Papaverine	9900 ± 600	0.48 ± 0.01
Benzodiazepines	(μM)	
Flunitrazepam	26 ± 2	0.75 ± 0.06
Nitrazepam	27 ± 4	0.97 ± 0.12
Diazepam	27 ± 4	0.83 ± 0.15
Diazepam Injection Solution USP	29 ± 2	0.94 ± 0.04
Desmethyldiazepam	33 ± 4	0.98 ± 0.10
Clonazepam	104 ± 11	0.91 ± 0.02
Oxazepam	117 ± 6	0.76 ± 0.11
Chlordiazepoxide	151 ± 40	0.79 ± 0.02
Lorazepam	176 ± 41	1.00 ± 0.03

* IC_{50} values were determined using 1.0 nM [^{3}H]NBMPR(stored blood). Results are the means ± s.e.mean from at least three experiments performed in duplicate.

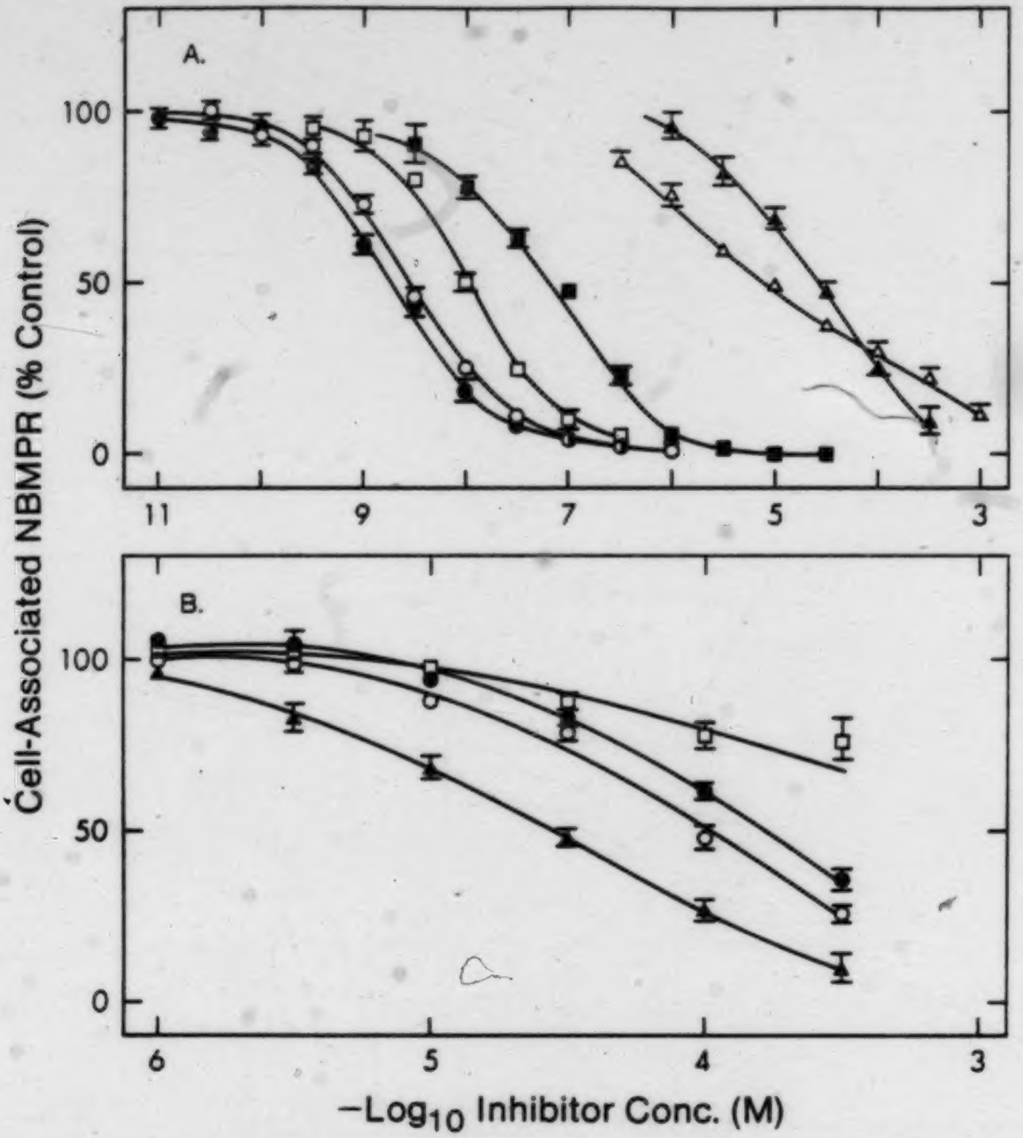


FIGURE 7 Inhibition of the site-specific binding of [^{3}H]NBMPR(1.0 nM, initial concentration) to stored human erythrocytes by recognized nucleoside transport inhibitors and benzodiazepines. Inhibitors: A. dilazep(●); nitrobenzylthioguanosine(○); dipyridamole(□); lidoflazine(■); papaverine(△); diazepam(▲). B. diazepam(▲); clonazepam(○); lorazepam (●); flurazepam(□). Each point represents mean ± s.e.mean.

TABLE 4 Inhibition of the site-specific binding of $[^3H]$NBMPR to fresh human erythrocytes by various benzodiazepine derivatives.

Inhibitor	% Inhibition at 300 μM	IC_{50}* (μM)	n_H
Ro 5-4864	100	14.3 ± 0.3	0.98 ± 0.01
Ro 11-6893(-)	95 ± 1	38 ± 3	1.07 ± 0.03
Midazolam	90 ± 1	51 ± 2	1.01 ± 0.02
Ro 11-6896(+)	51 ± 2	277 ± 9	0.77 ± 0.02
Flurazepam	27 ± 3		
Didesethylflurazepam	22 ± 2		
Ro 15-1788	20 ± 5		

* IC_{50} values were determined using 0.35 nM $[^3H]$NBMPR(fresh blood). Results are the means ± s.e.mean from at least three experiments performed in duplicate.

[^{3}H]NBMPR (Table 3, Figure 7B). Double reciprocal plot analysis of the inhibition of the binding of [^{3}H]NBMPR by Ro 5-4864, diazepam, clonazepam, chlordiazepoxide, and lorazepam indicated that the inhibition was apparently competitive in nature (Figure 6). The affinities of the benzodiazepines for the [^{3}H]NBMPR site were similar in erythrocytes isolated from both fresh and stored blood (Table 2). The benzodiazepines also appeared to interact with the [^{3}H]NBMPR site in an apparently non-cooperative manner since all benzodiazepine Hill coefficients were greater than 0.75 (Table 3 & 4). Flurazepam and its active metabolite, didesethylflurazepam, were very weak inhibitors of the site-specific binding of [^{3}H]NBMPR, as was the potent benzodiazepine antagonist Ro 15-1788; each produced approximately 25% inhibition at a concentration of 300 μM (Table 4). The interaction of benzodiazepines with the [^{3}H]NBMPR site appears to be influenced by the conformation at one benzodiazepine chiral centre because the affinity for the site of the (-) isomer, Ro 11-6893 (IC_{50} = 38 μM), was found to be 7-fold higher than that of the (+) isomer, Ro 11-6896 (IC_{50} = 277.3 μM) (Table 4). Several β-carboline derivatives were also very weak inhibitors of the binding of [^{3}H]NBMPR (Table 5).

Specificity of the inhibition of the binding of [^{3}H]NBMPR was tested by examining the effect of 300 μM concentrations of various compounds (agonists and antagonists for other biological receptor systems and agents which produce nonspecific membrane effects) on the binding of [^{3}H]NBMPR to human erythrocytes. These included acetylsalicylic acid, 2-chloroadenosine, cimetidine, cocaine, corticosterone, fentanyl, flurbiprofen, haloperidol, histamine, hydrocortisone, imipramine, ketamine, mepyramine, morphine, ouabain, pancuronium, pento-

TABLE 5 Inhibition of the site-specific binding of [^{3}H]NBMPR to human erythrocytes by β-carbolines and miscellaneous compounds

Compound	% Inhibition at 300 μM*
β-Carbolines	
Norharmane-3-carboxylic acid ethyl ester	53 ± 2
Harmine	47 ± 4
D-1,2,3,4-Tetrahydro-norharmane carboxylic acid ethyl ester	45 ± 1
L-1,2,3,4-Tetrahydro-norharmane carboxylic acid ethyl ester	40 ± 2
Harmaline	39 ± 4
Harmal	36 ± 1
Others	
Corticosterone	68 ± 2
2-Chloroadenosine	48 ± 2
Quinidine	39 ± 6
Haloperidol	34 ± 1
Pentobarbitol	29 ± 3
Flurbiprofen	23 ± 3
Phenytoin	16 ± 3
Pancuronium	15 ± 6

* % Inhibition values were determined using 0.35 nM [^{3}H]NBMPR in fresh human erythrocytes or 1.0 nM [^{3}H]NBMPR in stored human erythrocytes.

Results are the means ± s.e.mean from three experiments performed in duplicate.

barbitol, phentolamine, phenytoin, propranolol, quinidine, theophylline, thiopental, and tubocurarine. Of these, only corticosterone, 2-chloroadenosine, quinidine, haloperidol, pentobarbitol, flurbiprofen, phenytoin, and pancuronium inhibited the site-specific binding of [^{3}H]NBMPR (Table 5). Similarly, adrenaline, *gamma*-aminobutyric acid, glucose, glycine, proline, and tryptophan did not affect the binding of [^{3}H]NBMPR at concentrations up to 10 mM. The solvent, dimethyl sulfoxide (DMSO), at concentrations present in assay mixtures (not greater than 3%), did not influence the site-specific binding of [^{3}H]NBMPR.

3) Effect of ligand ([^{3}H]NBMPR) depletion:

a) General comments: Data obtained from [^{3}H]NBMPR binding assays were analysed using two procedures, one of which used the initial assay concentrations of [^{3}H]NBMPR in all calculations, and the other used the final, free concentrations of [^{3}H]NBMPR remaining following the attainment of equilibrium. Final, free concentrations of [^{3}H]NBMPR were determined by subtracting the concentrations of site-bound [^{3}H]NBMPR from the corresponding initial (prior to initiation of binding assays) concentrations of [^{3}H]NBMPR. These final, free concentrations are the 'depletion corrected' concentrations of [^{3}H]NBMPR. Depletion of [^{3}H]NBMPR was considered to be significant when more than 10% of the initial concentration of [^{3}H]NBMPR became site-bound at equilibrium. Doubling the number of cells used in the binding assays would be expected to double the degree of [^{3}H]NBMPR depletion. Similarly, the concentration of inhibitor present in competition experiments would also be subject to depletion via inhibitor binding to both specific and nonspecific sites. In order to determine the influence of depletion on apparent [^{3}H]NBMPR binding constants and apparent dipyridamole inhibition constants, binding assays were conducted using either 1.5 X 10^7 cells/ml or 3.0 X 10^7 cells/ml, and with or without correction for [^{3}H]NBMPR depletion.

b) Influence of [^{3}H]NBMPR depletion on [^{3}H]NBMPR binding constants: With correction for [^{3}H]NBMPR depletion, doubling the cell concentration used in the assays did not significantly affect the apparent [^{3}H]NBMPR binding constants. However, without correction for [^{3}H]NBMPR depletion, K_D values for [^{3}H]NBMPR were 2-fold higher when using 1.5 X 10^7 cells/ml and almost 5-fold higher when using 3.0 X 10^7 cells/ml than the corresponding values obtained from 'depletion corrected' data. Similarly, the B_{max} values for [^{3}H]NBMPR were overestimated by almost 2-fold if data obtained using 3.0 X 10^7 cells/ml were used without first being corrected for [^{3}H]NBMPR depletion (Table 6).

c) Influence of [^{3}H]NBMPR depletion on dipyridamole inhibition constants: With correction for [^{3}H]NBMPR depletion, dipyridamole appeared to be about twice as effective as inhibitor of the binding of [^{3}H]NBMPR when binding assays were conducted using 1.5 X 10^7 cells/ml than when 3.0 X 10^7 cells/ml were used. Furthermore, the apparent nature of the inhibition of the binding of [^{3}H]NBMPR by dipyridamole changed from apparently competitive when using 1.5 X 10^7 cells/ml to apparently noncompetitive when using 3.0 X 10^7 cells/ml in the binding assays (Table 6, Figure 8). These results may be due to depletion of dipyridamole from the assay medium, a phenomenon which could not be corrected for in the present experiments.

Without correction for [3H]NBMPR depletion, dipyridamole inhibition

TABLE 6 Effect of [^{3}H]NBMPR and dipyridamole depletion on the [^{3}H]NBMPR binding parameters(K_D and B_{max}) and dipyridamole inhibition parameters(K_i and type of inhibition) in fresh human erythrocytes

# of cells per assay		Corrected for [^{3}H]NBMPR depletion	NOT corrected for [^{3}H]NBMPR depletion
1.5 X 10^7	*K_D	0.24 ± 0.04 (3)	0.51 ± 0.01 (3)
	†B_{max}	11800 ± 400 (3)	13800 ± 400 (3)
	*K_i	1.9 ± 0.4 (9)	3.3 ± 0.6 (9)
	Type	Competitive	Competitive/Mixed
3.0 X 10^7	K_D	0.37 ± 0.3 (3)	1.75 ± 0.28 (3)
	B_{max}	15600 ± 2200 (3)	25400 ± 3300 (3)
	K_i	3.4 ± 0.3 (6)	10.5 ± 1.7 (6)
	Type	Noncompetitive	Noncompetitive/ Uncompetitive

Results are the means ± s.e.mean from the number of experiments indicated in parentheses.

* All K_D and K_i values shown are nM concentrations.

† B_{max} values are given as molecules of [^{3}H]NBMPR site-bound per cell.

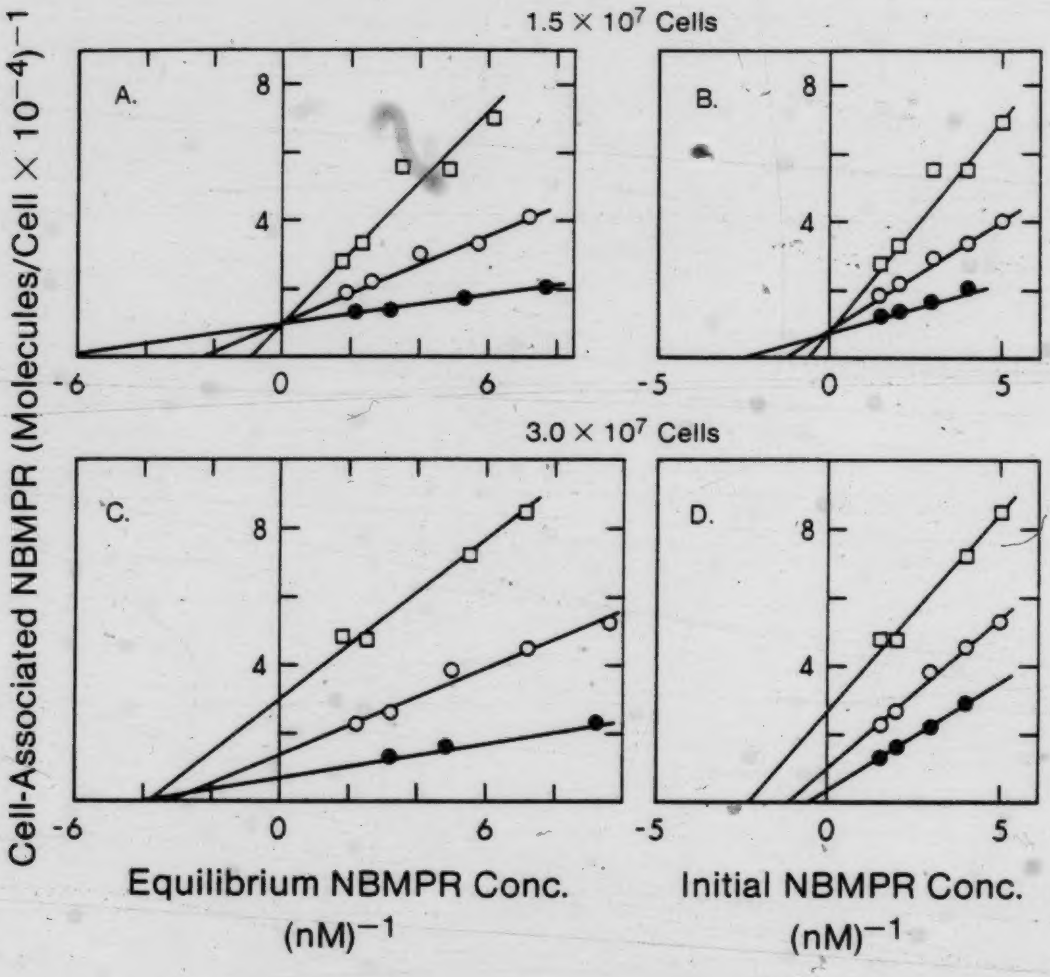


FIGURE 8 Double reciprocal plot analysis of the inhibiton of the site-specific binding of [^{3}H]NBMPR in fresh human erythrocytes by dipyridamole: Effect of [^{3}H]NBMPR and dipyridamole depletion from the assay medium. Assays were conducted at 22°C using concentrations of dipyridamole of 0 nM(●), 1 nM(○) and 3 nM(□). Either 1.5 X 10^7 cells/ml(A,B) or 3.0 X 10^7 cells/ml(C,D) were used in the binding assays, with(A,C) and without(B,D) correction for [^{3}H]NBMPR depletion.

constants were 2-fold and 3-fold higher when using 1.5 X 10^7 cells/ml and 3.0 X 10^7 cells/ml in the assay, respectively, compared to the corresponding values obtained with 'depletion corrected' data. Also, without correction for [^{3}H]NBMPR depletion, the nature of the inhibition of the binding of [^{3}H]NBMPR by dipyridamole appeared to be of a mixed type (i.e., decreased B_{max} values and increased K_D values for [^{3}H]NBMPR in the presence of dipyridamole) when using 1.5 X 10^7 cells/ml in the assay. On the other hand, when using 3.0 X 10^7 cells/ml without 'depletion correction', dipyridamole appeared to inhibit the binding of [^{3}H]NBMPR in an uncompetitive manner (decreased B_{max} values for [^{3}H]NBMPR with no change in K_D in the presence of dipyridamole) (Table 6, Figure 8).

Note: Data presented in Table 6 and Figure 8 were obtained using erythrocytes from fresh blood, but comparable data were obtained when erythrocytes from stored blood were used.

4) Thermodynamic and kinetic analysis of [^{3}H]NBMPR and inhibitor interactions with the [^{3}H]NBMPR binding site:

a) Thermodynamic analysis: Measurement of the equilibrium dissociation constant of [^{3}H]NBMPR for its sites in human erythrocytes revealed that the affinity of [^{3}H]NBMPR increased as the incubation temperature was reduced from 37° to 4°C (Table 7). This may be due to the slower dissociation rate of [^{3}H]NBMPR from its sites at the lower temperatures (*vide infra*). Changing the incubation temperature did not affect the maximum number of [^{3}H]NBMPR binding sites. The interaction of adenosine with these sites showed a similar temperature dependence to that for the binding of [^{3}H]NBMPR (Table 7). Conversely, the affinity

TABLE 7 Effect of incubation temperature on the dissociation constant (K_D) of [^{3}H]NBMPR and the inhibition constants(K_i) of several inhibitors of the site-specific binding of [^{3}H]NBMPR in fresh human erythrocytes

Compound	Incubation Temperature(°C)					
	3-4	10	15-16	22-23	29-30	35-39
[^{3}H]NBMPR K_D(nM)	0.08	0.10	0.12	0.24	0.39	0.62
Dipyridamole K_i(nM)	9.4	14	5.0	4.7	5.1	3.0
Adenosine K_i (μM)	14	-	57	115	197	154
Diazepam K_i (μM)	31	40	5.2	8.5	13	5.4

Results are the means from at least four experimental determinations.

of dipyridamole and diazepam for the [^{3}H]NBMPR site decreased as the temperature was reduced (Table 7). Plots of ln K_A against 1/T°K (van't Hoff plots) were linear (Figure 9) and $T\Delta S$ values derived from these plots (Table 8) were similar to those calculated from the relationship $\Delta G = \Delta H - T\Delta S$. The interaction of [^{3}H]NBMPR or adenosine with [^{3}H]NBMPR sites resulted in a decrease in enthalpy (ΔH negative), whereas dipyridamole or diazepam interaction with these sites produced an increase in enthalpy (ΔH positive) (Table 8). The dipyridamole and diazepam binding reactions also significantly increased entropy within the system (ΔS positive). [^{3}H]NBMPR, however, produced a much smaller change in entropy than did dipyridamole and diazepam (Table 8). The adenosine site-interaction resulted in an actual decrease in entropy (ΔS negative). All interactions with the [^{3}H]NBMPR binding complex resulted in a decrease in Gibbs free energy (ΔG negative) (Table 8).

b) Kinetic analysis: The rate of dissociation of site-bound [^{3}H]NBMPR in human erythrocytes was measured utilizing nonradioactive NBMPR (300 nM) to prevent reassociation of free [^{3}H]NBMPR ('displacer'). Several other inhibitors, or combinations of inhibitors, were also used as 'displacers'. These included, dipyridamole (2 μM), uridine (100 mM), adenosine (10 mM), and diazepam (1 mM) in the presence and absence of nonradioactive NBMPR (300 nM). A semilogarithmic plot of the site-bound [^{3}H]NBMPR which remained at various times after the addition of 'displacer' indicated that the dissociation of [^{3}H]NBMPR followed first order kinetics (Figure 10). The rate constant, however, depended upon the 'displacer' used (Figure 10, Table 9). The rate of dissociation of [^{3}H]NBMPR induced by each 'displacer' at 22°C [illegible] independent of the presence of 300 nM nonradioactive NBMPR (compare rate constants in

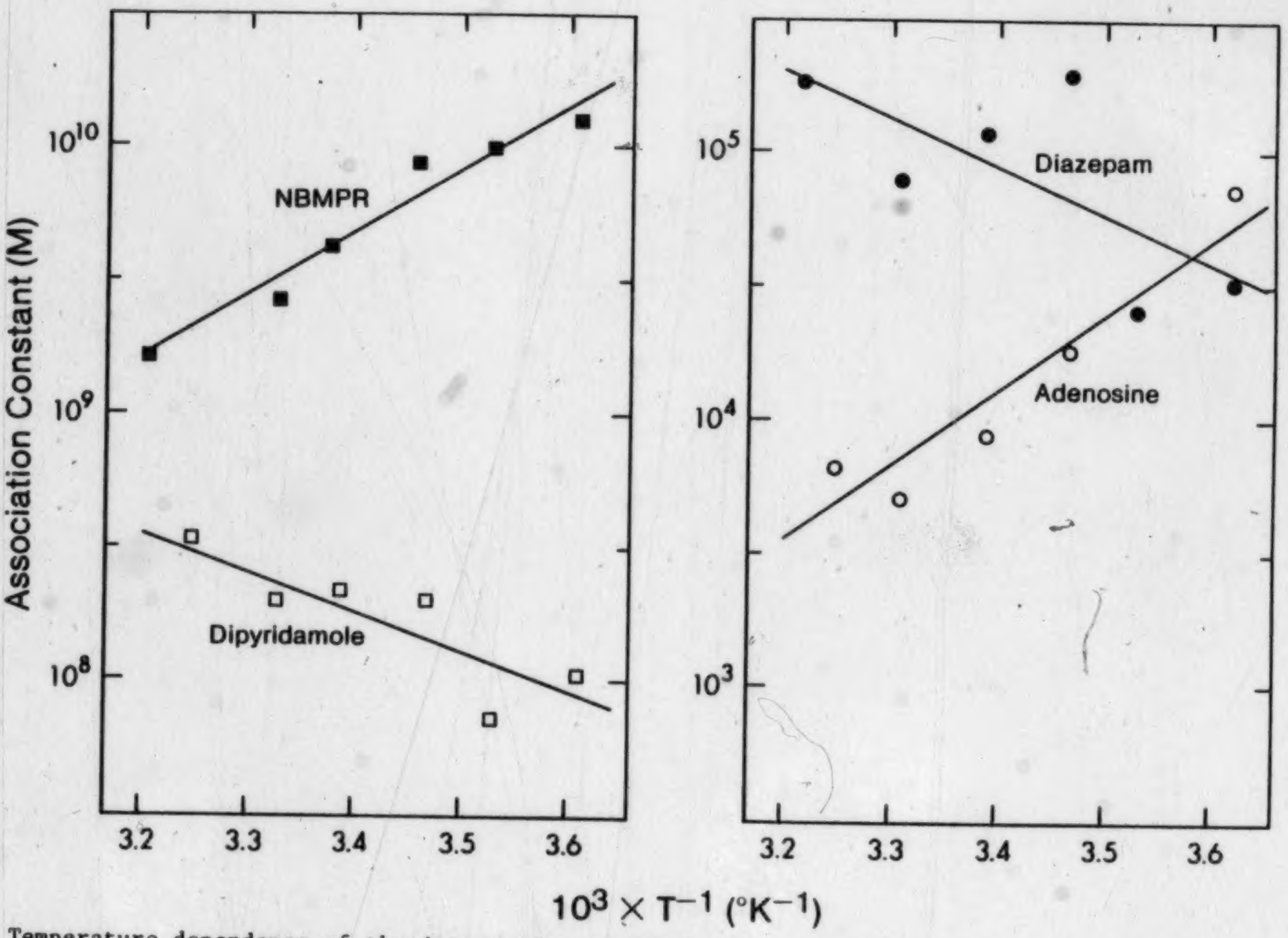


FIGURE 9 Temperature dependence of the interaction of NBMPR, dipyridamole, diazepam, and adenosine with [^{3}H]NBMPR binding sites in fresh human erythrocytes--Van't Hoff plots. Each point is the mean of at least three experiments.

TABLE 8 Thermodynamic constants for the interactions of $[^3H]$NBMPR, dipyridamole, diazepam, and adenosine with the $[^3H]$NBMPR binding sites in fresh human erythrocytes at 37°C

	ΔG^* (kJ/mole)	$\Delta H^\dagger$ (kJ/mole)	$T\Delta S^\dagger$ (kJ/mole)
$[^3H]$NBMPR	-54.7	-45.3	-0.2
Dipyridamole	-50.7	+28.0	+66.7
Diazepam	-28.1	+34.1	+53.3
Adenosine	-22.6	-54.7	-45.3

* ΔG values were calculated according to the relationship $\Delta G = RT \ln K_i$

† ΔH and ΔS values were determined from van't Hoff plots (Figure 9) as described in Section F.2.g. of Methods.

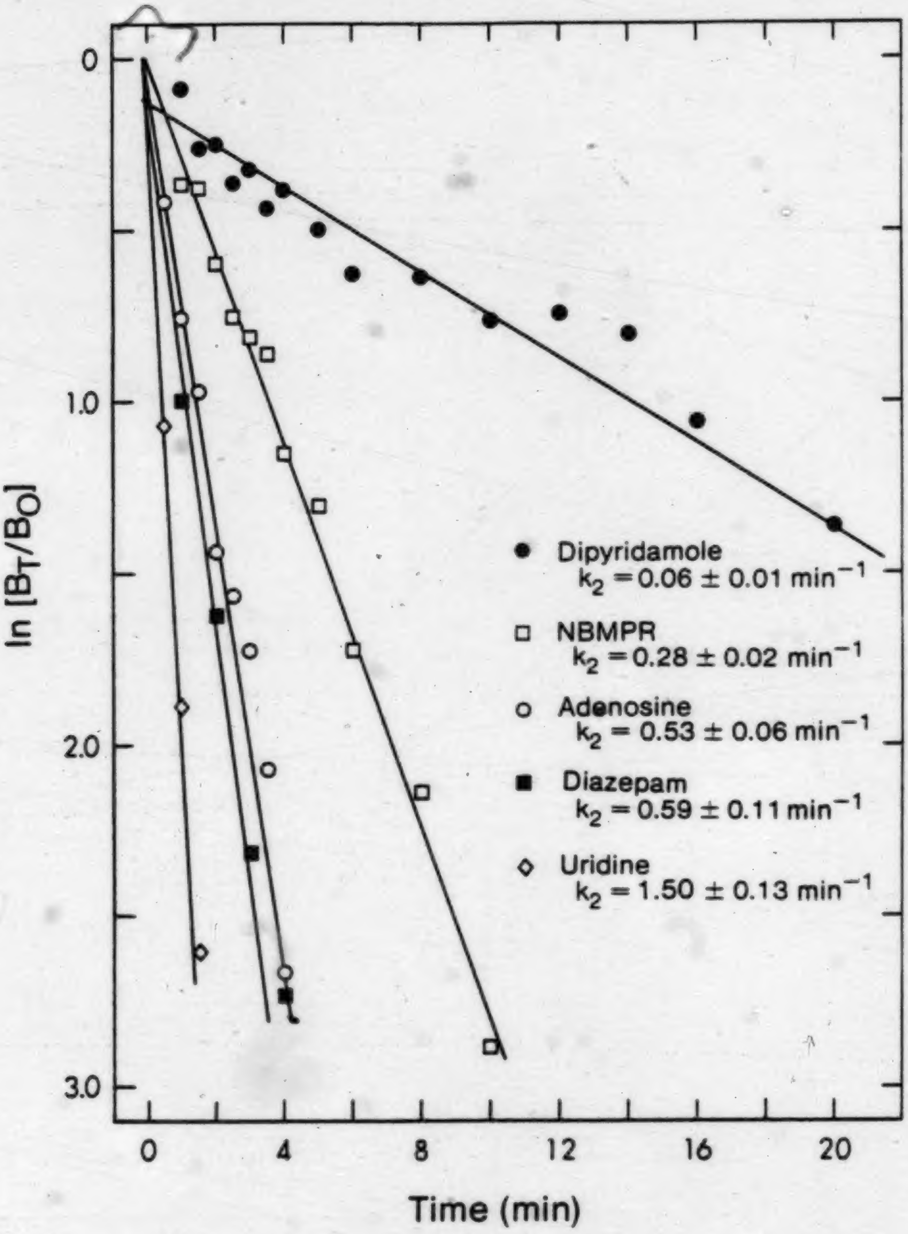


FIGURE 10 Dissociation of site-bound [^{3}H]NBMPR from fresh human erythrocytes at 22°C in the presence of various 'displacers'. Each point is the mean of at least three experiments.

TABLE 9 Effect of incubation temperature on the dissociation rate constants(k_2 values) of [^{3}H]NBMPR which were obtained using nonisotopic NBMPR, alone and in combination with one of several other 'displacers', in fresh human erythrocytes

'Displacer'	Dissociation Rate Constants(k_2) at various incubation temperatures (min^{-1})						Activation Energy for Dissociation of [^{3}H]NBMPR (E_a-) (kJ/mole)
	4°C	9°C	16°C	22°C	26°C	37°C	
NBMPR(300 μM)	0.02	0.06	0.10	0.28	0.50	1.52	98 ± 12
+ Dipyridamole(2 μM)	0.003	0.02	0.03	0.06	0.11	0.60	106 ± 19
+ Adenosine(10 mM)	0.02	0.13	0.19	0.55	0.90	2.19	95 ± 22
+ Uridine(100 mM)	0.06	0.35	0.43	1.68	1.20	4.38	87 ± 30

k_2 values are the means from at least four experimental determinations.

E_a(-) values are the means ± s.e.mean(n = 6)

Incubation temperatures shown are the average temperatures of at least four experiments.

Figure 10 to those in Table 9). Dipyridamole produced the slowest rate of dissociation ($t_{1/2} \approx 9$ min at 22°C) followed by nonisotopic NBMPR, and then adenosine and diazepam. Uridine produced the most rapid rate of dissociation of [^{3}H]NBMPR ($t_{1/2} \approx 0.35$ min at 22°C) which was 25 times faster than that produced by dipyridamole.

The dissociation rate of [^{3}H]NBMPR from its sites in human erythrocytes was temperature dependent; the dissociation rate at 37°C was 70 to 200 times faster than that observed at 4°C, irrespective of the 'displacer' used (Figure 11, Table 9). Therefore, although each 'displacer' gave a different dissociation rate, the activation energy ($E_a(-)$) for the dissociation of [^{3}H]NBMPR from its sites in human erythrocytes, determined from the slope of the Arrhenius plot (Figure 12) was similar in each case (≈ 97 kJ/mole, Table 9).

B. Species Differences in the Binding of [^{3}H]NBMPR to Erythrocytes

Site-specific binding of [^{3}H]NBMPR was demonstrated in rabbit, mouse, and rat erythrocytes (Table 10). Rabbit erythrocytes appeared to be similar to human erythrocytes with respect to the number of [^{3}H]NBMPR binding sites per cell, but the affinity of [^{3}H]NBMPR for its sites in rabbit erythrocytes was almost 5 times lower than that in human erythrocytes (Table 10, Figure 13A). On the other hand, mouse and rat erythrocytes possessed sites which bound [^{3}H]NBMPR with an affinity similar to that determined in fresh human erythrocytes, but the densities of these sites in mouse and rat cells were 30% and 2.7%, respectively, of that of human cells (Table 10).

Diazepam and dipyridamole inhibited the site-specific binding of

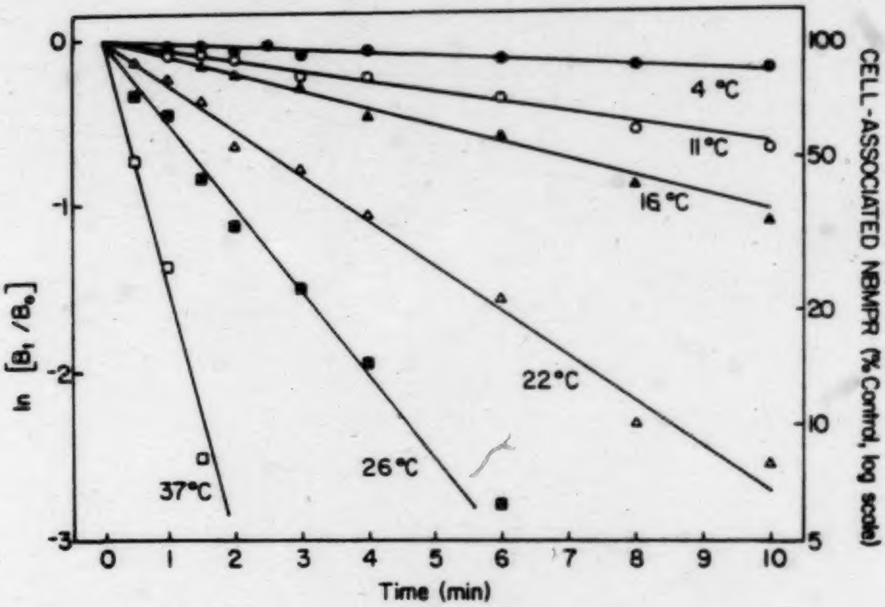
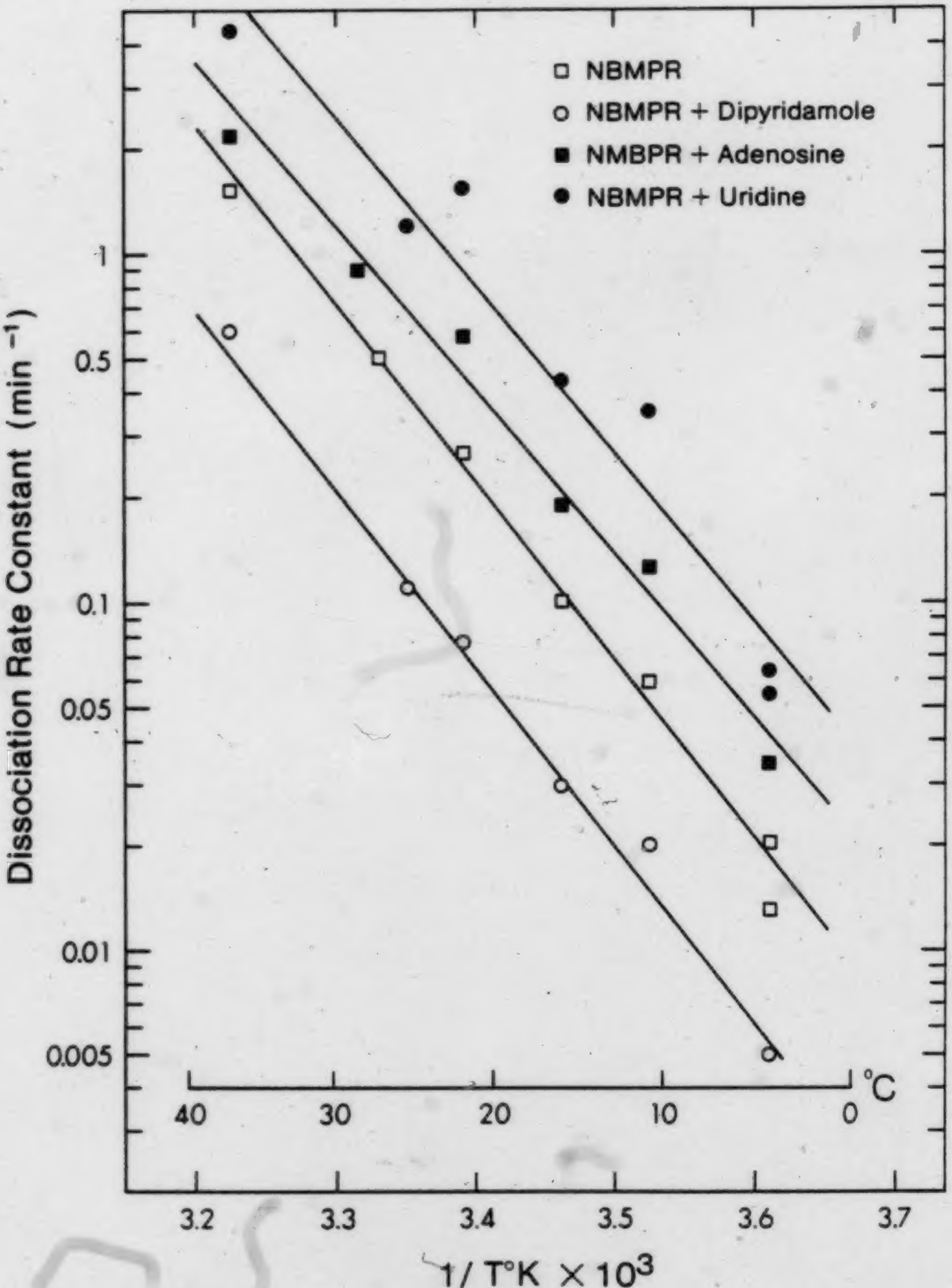


FIGURE 11 Effect of incubation temperature on the dissociation of site-bound [^{3}H]NBMPR from fresh human erythrocytes. Dissociation of site-bound [^{3}H]NBMPR was initiated by the addition of nonisotopic NBMPR(300 nM) to the assay medium.

FIGURE 12 Effect of incubation temperature on the dissociation rate constants of [^{3}H]NBMPR determined using fresh human erythrocytes -- Arrhenius plot. Data were obtained using nonradioactive NBMPR, alone, and in combination with one of several other 'displacers', as indicated. Each point is the mean of at least four experiments.



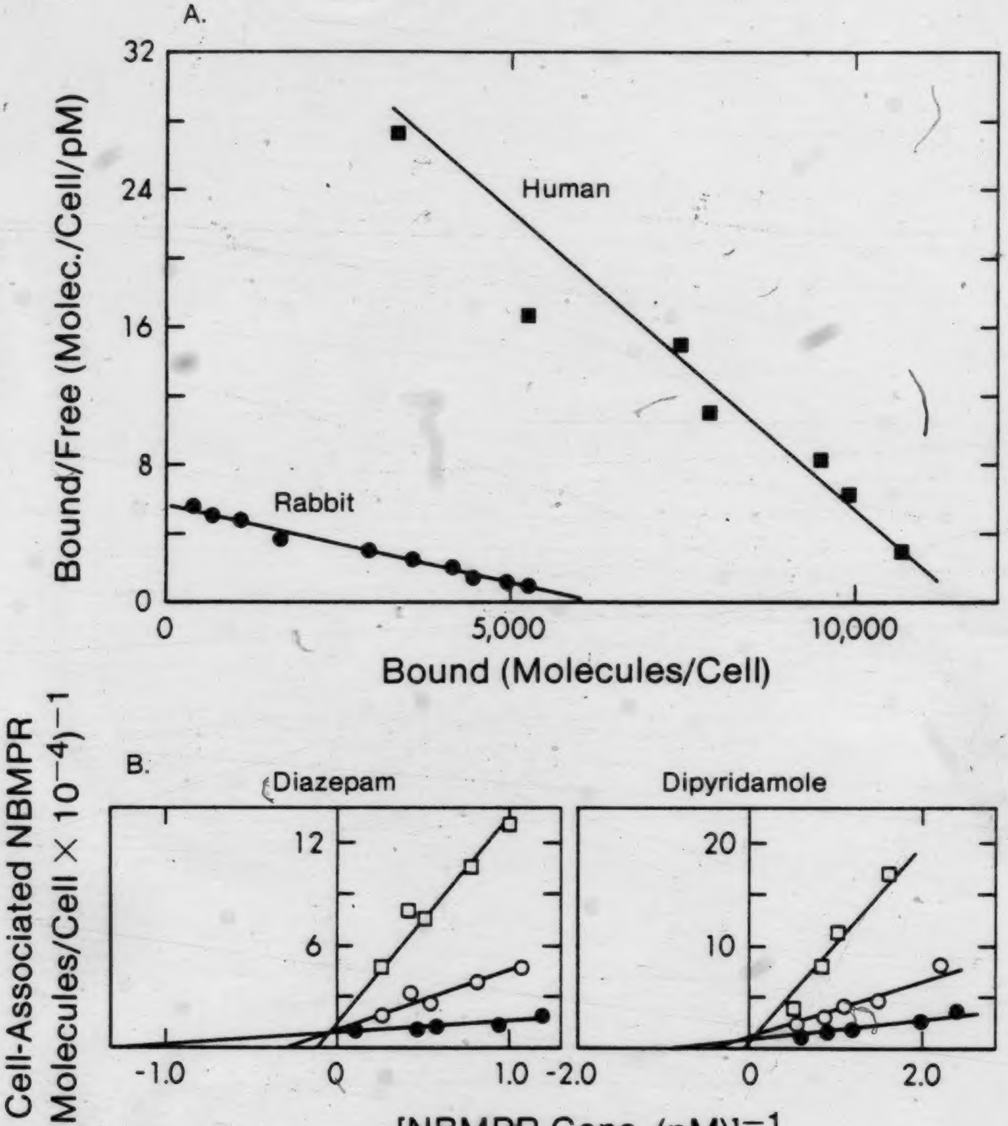
□ NBMPR
○ NBMPR + Dipyridamole
■ NMBPR + Adenosine
● NBMPR + Uridine
Dissociation Rate Constant (min ⁻¹)
1
0.5
0.1
0.05
0.01
0.005
40
30
20
10
0
°C
3.2
3.3
3.4
3.5
3.6
3.7
1/ T°K × 10³

TABLE 10 Species differences in the site-specific binding of $[^3H]$NBMPR to mammalian erythrocytes

Species	B_{max} (molecules/cell)	K_D (nM)
Human (9)	11000 ± 600	0.31 ± 0.02
Rabbit (8)	9500 ± 800	1.4 ± 0.1
Mouse (4)	3300 ± 500	0.14 ± 0.02
Rat (6)	300 ± 30	0.28 ± 0.11
Dog (4)	not detectable	

Results are the means ± s.e.mean from the number of experiments indicated in parentheses.

FIGURE 13 Site-specific binding of [^{3}H]NBMPR to fresh rabbit erythrocytes and its inhibition by diazepam and dipyridamole. A. Representative Scatchard plot comparing the binding of [^{3}H]NBMPR to fresh rabbit and fresh human erythrocytes. B. Double reciprocal plot analysis of the inhibition of the site-specific binding of [^{3}H]NBMPR to rabbit erythrocytes by diazepam(0 μM, ● ; 30 μM, ○ ; 100 μM, □) and dipyridamole(0 nM, ● ; 10 nM, ○ ; 30 nM, □)


A.
Human
Rabbit
Bound/Free (Molec./Cell/pM)
0
8
16
24
32
0
5,000
10,000
Bound (Molecules/Cell)
B.
Diazepam
Dipyridamole
Cell-Associated NBMPR
(Molecules/Cell × 10^{-4})$^{-1}$
12
6
20
10
-1.0
0
1.0
-2.0
0
2.0
[NBMPR Conc. (nM)]$^{-1}$

[^{3}H]NBMPR to rabbit erythrocytes in an apparently competitive manner (Figure 13B) with K_i values of 8.9 ± 2.0 M(4) and 4.6 ± 0.9 nM(4), respectively. These were similar to their affinities for the [^{3}H]NBMPR binding sites in human erythrocytes (Table 2).

C. Characterization of the Binding of [^{3}H]NBMPR to Guinea Pig CNS Membranes:

1) Characteristics of membrane preparations: The protein yield (mg protein/g wet weight tissue) and the % of total protein of each membrane fraction was determined (Table 11). The amount of protein associated with the microsomal fraction (supernatant of P_2 fraction) was estimated by subtracting the summed protein associated with P_1 and P_2 fractions from the protein content of the crude homogenate. This constituted about 32% of the total protein in the homogenate (Table 11). Most of the protein appeared to be associated with the P_2 fraction (41%), and 35% of the P_2 fraction was estimated to be synaptosomal-like material (collected from the 0.8/1.2 M interface of the sucrose gradient). The remainder of the P_2 fraction consisted of myelin and mitochondria. Approximately 77% of the protein associated with the P_2 fraction was recovered from the sucrose gradient as three distinct bands. Another 15% of the protein was recovered from the interband areas and the remaining 8% was probably lost during transfer and subsequent centrifugation steps (See Methods, Section C.4). The protein yields shown in Table 11 agree well with data obtained by Marchbanks (1975), using a similar procedure.

2) Properties of the binding of [^{3}H]NBMPR to CNS membranes: The

TABLE 11 Protein yield of each membrane fraction prepared from guinea pig cortical membrane homogenates by differential centrifugation and sucrose density centrifugation, as described in Methods.

Membrane Fraction	mg protein per g wet wt. cortex	% of homogenate
Homogenate	83 ± 8	100
P_1	22 ± 1	27 ± 3
P_2	34 ± 3	41 ± 4
Subfractionation of P_2		% of P_2
Myelin	10 ± 1	29 ± 1
Synaptosomes	12 ± 1	35 ± 2
Mitochondria	4.3 ± 0.4	13 ± 2

Each value represents the mean ± s.e.mean from three cortical membrane preparations.

site-specific binding of $[^3H]$NBMPR to guinea pig cortical membranes was proportional to the protein concentration in the assay mixtures over the range of 0.05 to 0.50 mg protein/ml (Figure 14A). Final protein concentrations in all binding assays ranged between 0.20 and 0.45 mg/ml. Site-specific binding of $[^3H]$NBMPR possessed a broad pH optimum with peak binding occurring in the range of 6.5 to 10 (Figure 14B).

Figure 15A shows the time-course of the binding of $[^3H]$NBMPR to cortical synaptosomes at 22°C. The specific binding of 0.35 nM $[^3H]$NBMPR occurred rapidly, was 50% complete after approximately 1.5 min, and binding reaction equilibrium was attained by 10 min. Increasing the concentration of $[^3H]$NBMPR to 1.0 nM resulted in an association rate which was significantly higher than that of 0.35 nM $[^3H]$NBMPR; the binding was 50% complete after approximately 1.2 min with equilibrium being attained by 10 min. The observed forward rate constants (k_{ob}), calculated from the slope of the lines in Figure 15B, were 0.29 ± 0.06 $\min^{-1}$ for 0.35 nM $[^3H]$NBMPR, and 0.66 ± 0.12 $\min^{-1}$ for 1.0 nM $[^3H]$NBMPR. This allows calculation of the association (k_1) and dissociation (k_2) rate constants from the relationship $k_{ob} = k_1[^3\text{H-NBMPR}] + k_2$; values of 0.56 and 0.09, respectively, were obtained. An estimate of the equilibrium dissociation constant (K_D), obtained as the ratio k_2/k_1, was 0.16 nM. When binding assays were conducted at 4°C, $[^3H]$NBMPR associated with its specific sites in guinea pig cortical membranes at a much slower rate than that observed at 22°C. At 4°C, site-specific binding of $[^3H]$NBMPR (0.35 nM) was 50% complete after approximately 5 min and equilibrium was attained by 60 min (Figure 16). The observed forward rate constant (k_{ob}) at 4°C was 0.052 ± 0.003 $\min^{-1}$.

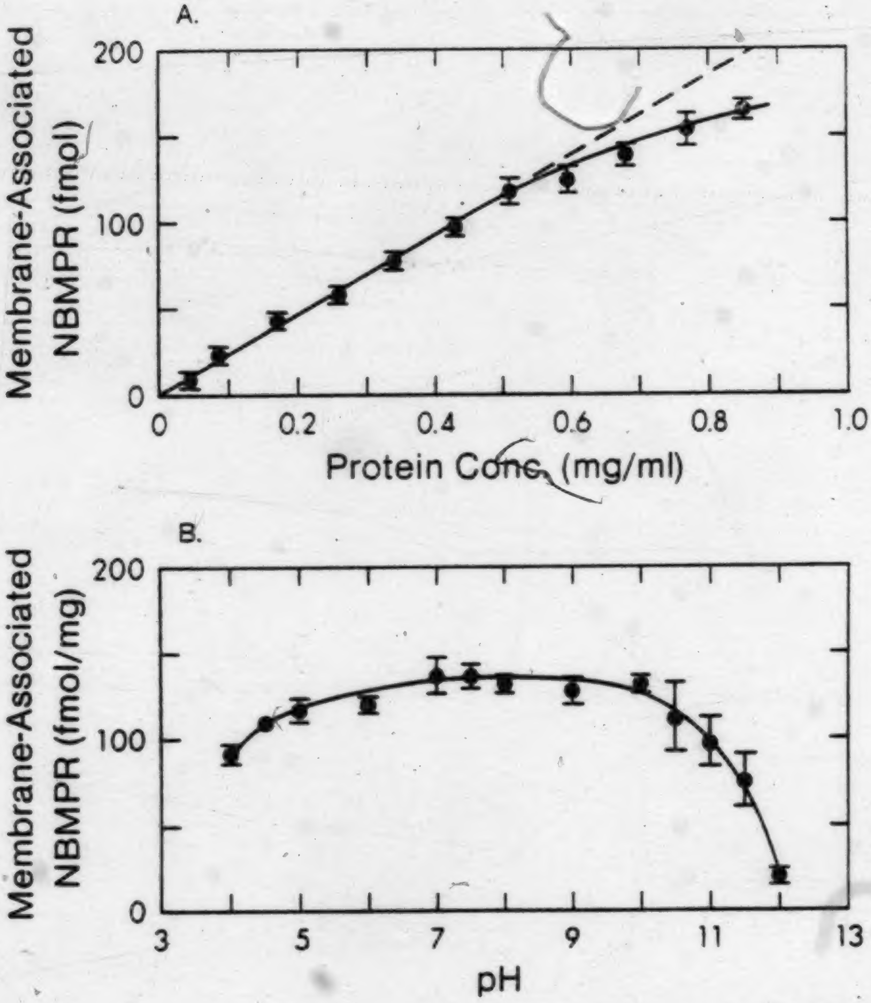


FIGURE 14 Effect of protein concentration(A) and pH of the incubation medium(B) on the site-specific binding of [^{3}H]NBMPR(1.0 nM) to guinea pig cortical membranes. Each point is the mean ± s.e.mean from three experiments performed in duplicate.

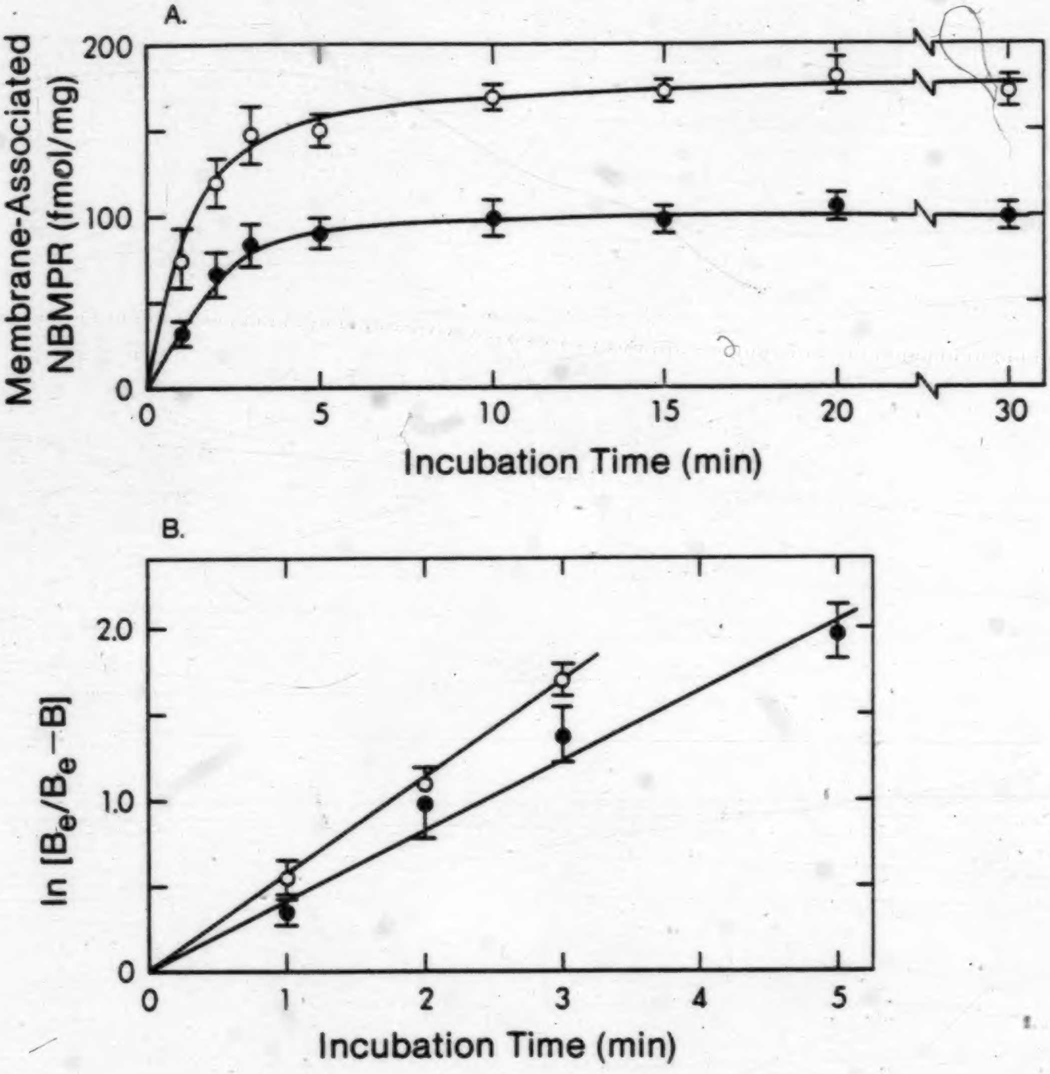


FIGURE 15 Time-courses for association of [^{3}H]NBMPR (0.35 nM, ●; 1.0 nM, ○, initial concentrations) to guinea pig cortical membranes at 22°C. B. is a psuedo first-order plot of the data shown in A., the slope of which provides an estimate of the observed forward rate constant (k_{ob}). Each point is the mean ± s.e.mean of three experiments performed in duplicate.

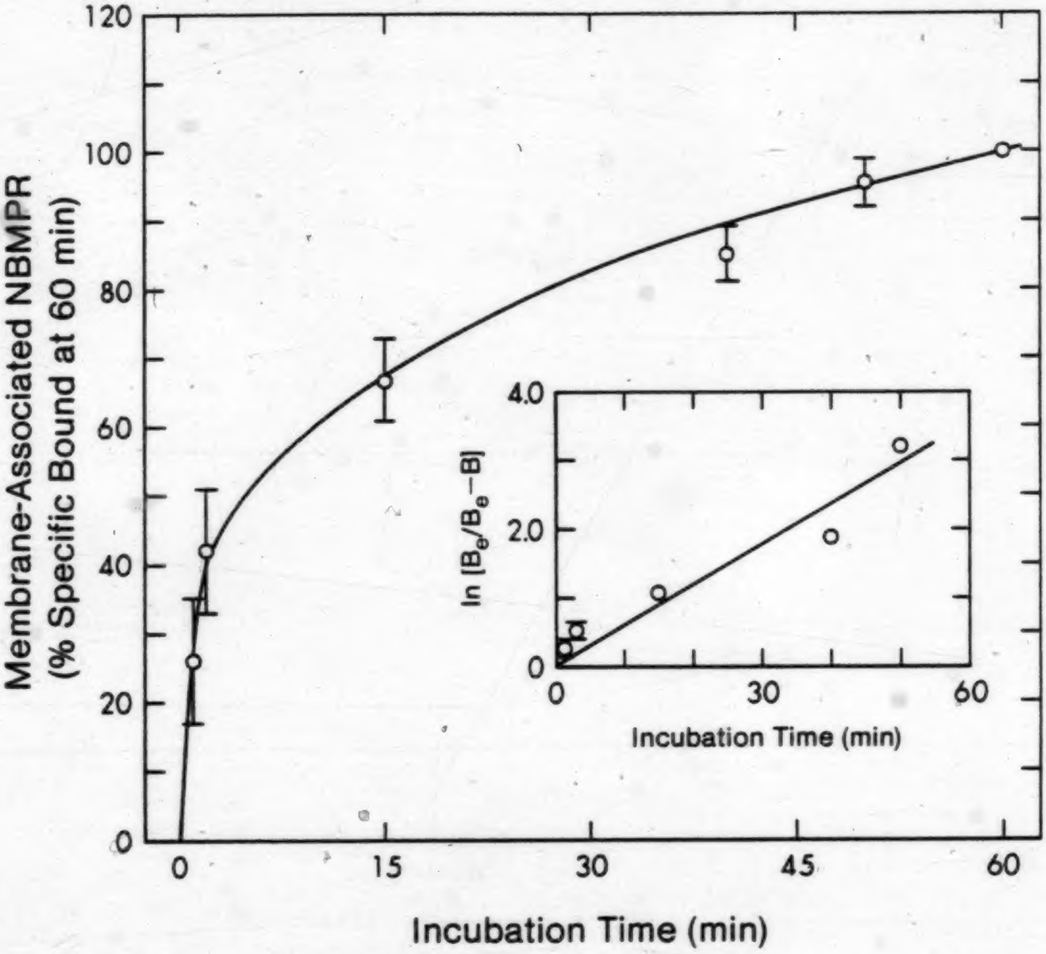


FIGURE 16 Time-course for association of [^{3}H]NBMPR(0.35 nM, initial concentration) to guinea pig cortical membranes at 4°C. The inset represents a pseudo first-order plot of this data, the slope of which provides an estimate of the observed forward rate constant(k_{ob}). Each point is the mean ± s.e.mean of three experiments performed in duplicate.

The 'total' amount of [^{3}H]NBMPR that became associated at equilibrium with cortical synaptosomes under standard assay conditions (pH 7.4, 22°C, 20 min incubation) consisted of two components (Figure 17), one of which was site-specific (displaceable by nonradiolabelled NBMPR, or other transport inhibitors) and saturable, whereas the other was proportional to free [^{3}H]NBMPR concentration in the assay medium. Mass law analysis (Scatchard plot) of such binding data (Figure 18A) yielded straight line plots which indicated that binding sites were of an apparent single type with a maximum binding capacity (B_{max}) for [^{3}H]NBMPR of approximately 300 fmol/mg protein. The apparent K_D of [^{3}H]NBMPR at these sites varied depending on the type of assay procedure employed: 0.25 nM when assays were terminated by centrifugation, or 0.10 nM when assays were terminated by filtration (Table 12, Figure 18A). These values are in agreement with that (0.16 nM) calculated from the kinetic experiments described above. Hill plots of the saturation data were linear (Figure 18B) with Hill coefficients which were not different from unity (Table 12). Storage of the membrane preparation for 24 h at 4°C resulted in [^{3}H]NBMPR binding constants (at 22°C) which were not significantly different from those obtained using freshly prepared membranes.

Preincubation of cortical synaptosomes with adenosine deaminase (70 μg/ml, final concentration) did not influence [^{3}H]NBMPR binding constants, suggesting that levels of extracellular adenosine in the incubation mixture did not influence the binding reaction. Also, the absence of sodium, potassium, magnesium, or calcium ions from the incubation medium did not affect the binding of [^{3}H]NBMPR.

Exposure of the synaptosomal suspension to a hypotonic medium (distilled water) apparently destroyed 36% of the specific [^{3}H]NBMPR

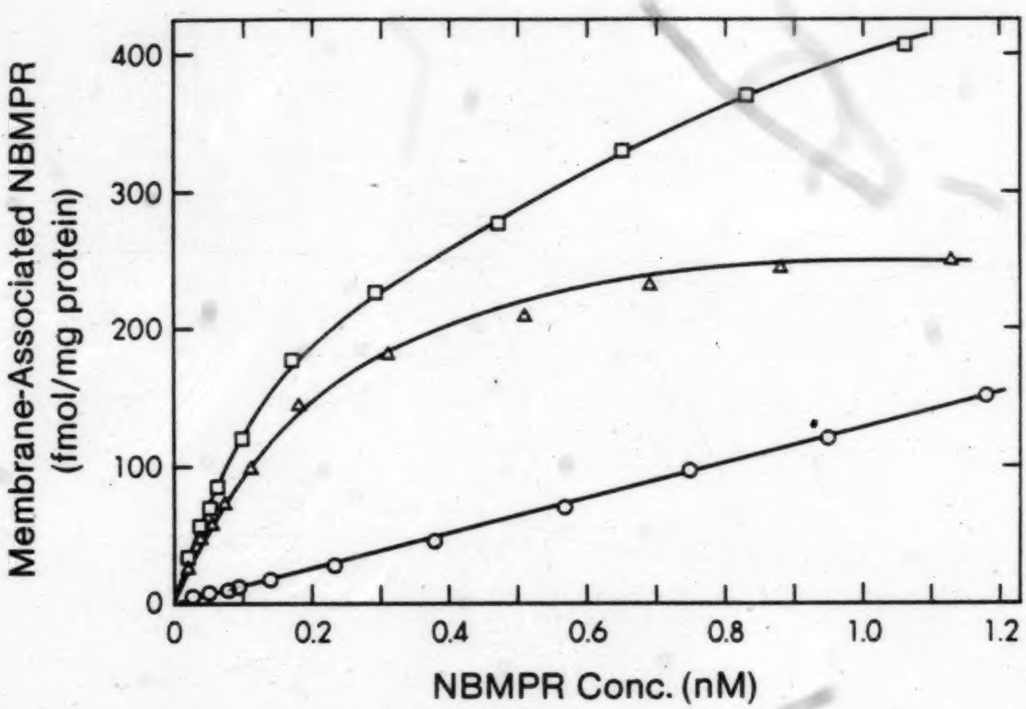


FIGURE 17 Concentration dependence of the binding of [^{3}H]NBMPR to guinea pig cortical membranes. This is a representative plot which is not different from those obtained from 6 other experiments. Total binding(□); nonspecific binding(○); specific binding(△).

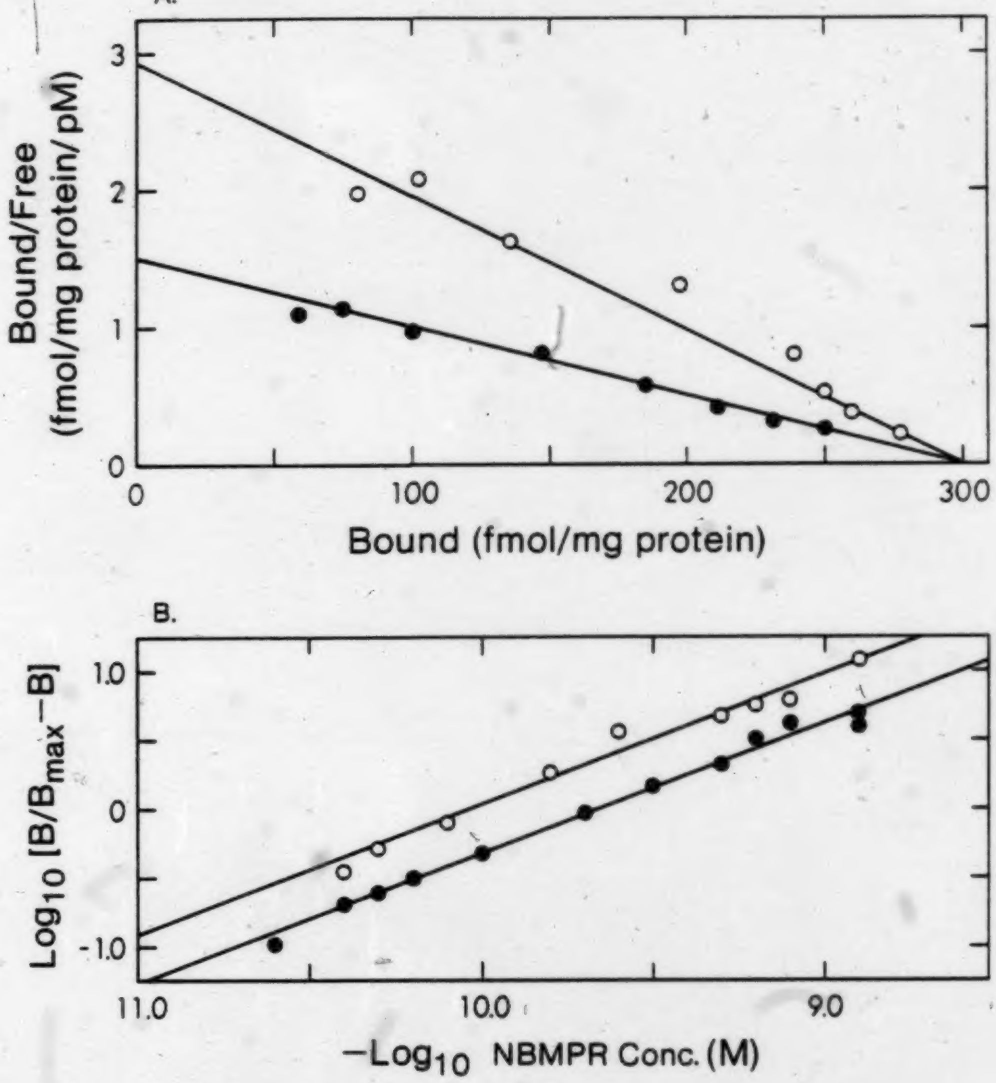


FIGURE 18 Scatchard(A) and Hill(B) plots of the site-specific binding of [^{3}H]NBMPR to guinea pig cortical membranes. Data were obtained using either a centrifugation assay(●) or a filtration assay(○) procedure.

TABLE 12 [^{3}H]NBMPR binding to specific sites in guinea pig cortical membranes: Effect of the assay termination procedure on the apparent binding parameters of [^{3}H]NBMPR.

Assay Methodology	B_{max} (fmol/mg protein)	K_D (nM)	n_H
Centrifugation	300 ± 10	0.25 ± 0.01	1.04 ± 0.02
Filtration	259 ± 15	0.10 ± 0.01	0.99 ± 0.03

Results are the means ± s.e.mean from three experiments performed in duplicate.

binding sites (determined by filtration assay) in these membranes without significantly affecting the affinity of the remaining sites for [^{3}H]NBMPR (Table 13, Figure 19).

Incubation of the cortical synaptosomes at 60°C for 1 h abolished 77% of the site-specific binding of [^{3}H]NBMPR and similarly, preincubation with trypsin (10 μg/ml, final concentration) destroyed 84% of the specific binding sites for [^{3}H]NBMPR (Table 13, Figure 19). Hill coefficients for the binding of [^{3}H]NBMPR to its sites remaining after heating, or treatment with trypsin were not different from unity and the affinity of [^{3}H]NBMPR for the remaining sites was similar to that for untreated synaptosomes (Table 13).

Analysis of the temperature dependence of the binding reaction revealed that the K_D of [^{3}H]NBMPR for its binding sites increased as the temperature of the incubation medium was raised (Table 14). This was probably due to an acceleration of the dissociation rate at the higher temperatures. The enthalpy (ΔH) and entropy ($T\Delta S$ at 37°C) changes associated with the binding reaction were determined from the van't Hoff equation to be 43 kJ/mole and 11 kJ/mole, respectively. These values are similar to those determined in human erythrocytes. The apparent maximum number of specific binding sites (B_{max}) for [^{3}H]NBMPR in guinea pig cortical synaptosomes increased slightly from 4°C to 37°C, and then fell sharply at 55°C, possibly due to protein denaturation (Table 14). A temperature of 22°C was considered optimal for binding assays as the ratio of specific to nonspecific binding was maximal at this temperature.

Estimates of the subcellular distribution of [^{3}H]NBMPR binding sites in guinea pig cortex homogenates indicated that sites possessed similar affinities and were present in similar densities in each fract-

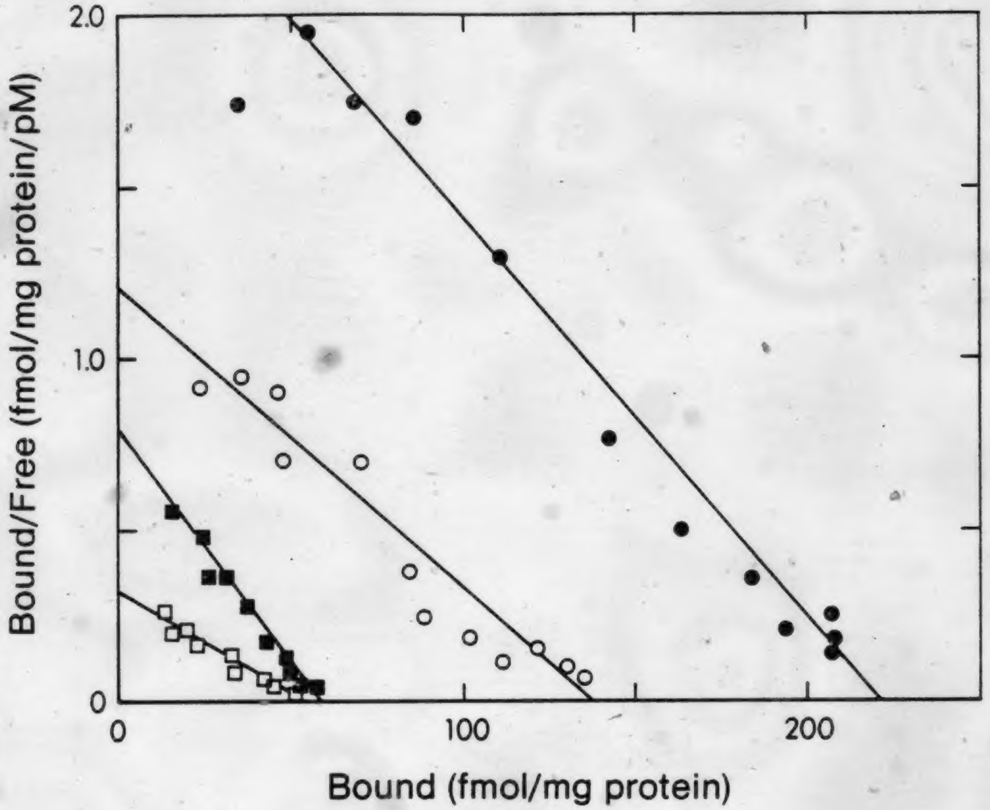


FIGURE 19 Effect of trypsin, high temperature(60°C) and osmotic shock on the site-specific binding of [^{3}H]NBMPR to guinea pig cortical membranes. Normal cortical membranes(intact synaptosomes)(●); osmotically shocked cortical membranes (lysed synaptosomes)(○); membranes heated at 60°C for 1 h(■); membranes preincubated with trypsin(10 μg/ml)(□). Data were obtained using a filtration assay procedure. The Scatchard plots shown are representative of those obtained from 3 similar experiments.

TABLE 13 Effect of various membrane treatments on the site-specific binding of [^{3}H]NBMPR to guinea pig cortical membranes.

Membrane Treatment	B_{max} (fmol/mg protein)	K_D (nM)	n_H
Intact Synaptosomes*	259 ± 15	0.10 ± 0.01	0.99 ± 0.03
Lysed Synaptosomes†	165 ± 16	0.15 ± 0.02	0.98 ± 0.04
60°C for 1 hour	59 ± 10	0.08 ± 0.01	0.82 ± 0.06
Trypsin (2 units/ml)	42 ± 7	0.18 ± 0.02	0.94 ± 0.09

* Intact synaptosomes as used in this context indicates that the membranes have not been subjected to any disruptive treatment; these are not necessarily intact vesicles.

† Lysed synaptosomes were prepared by vortexing 'intact synaptosomes' in water adjusted to a pH of 7.4.

Values shown are the means ± s.e.mean from three experiments performed in duplicate using filtration to terminate the binding assay.

TABLE 14 Effect of incubation temperature on the site-specific binding of [^{3}H]NBMPR to guinea pig cortical membranes.

Temperature (°C)	B_{max} (fmol/mg protein)	K_D (nM)
4	257 ± 12	0.10 ± 0.01
22	316 ± 8	0.22 ± 0.02
37	399 ± 19	0.77 ± 0.14
55	185 ± 3	1.20 ± 0.11

Results are the means ± s.e.mean from three experiments performed in duplicate using centrifugation to terminate the binding assay.

ion, and that this binding was not significantly different from that seen in the crude homogenate (Table 15). The site-specific binding of [^{3}H]NBMPR to the microsomal (P_3) fraction could not be determined by the centrifugation assay used here. Subfractionation of the crude synaptosomal fraction revealed that [^{3}H]NBMPR bound to similar numbers of sites in both myelin and purified synaptosomes. However, the mitochondrial fraction had a significantly lower density of sites (Table 15).

3) Inhibition of the binding of [^{3}H]NBMPR: Several nucleoside derivatives inhibited the binding of [^{3}H]NBMPR in an apparently competitive manner (Figure 20). Nonradioactive NBMPR inhibited the site-specific binding of [^{3}H]NBMPR with a K_i value of 0.22 nM, as determined by mass law analysis using the double reciprocal plot method (Table 16). This value is similar to the K_D (0.25 nM) of [^{3}H]NBMPR as determined by Scatchard analysis of the site-specific binding of [^{3}H]NBMPR. Nitrobenzylthioinosine phosphate (cleaved to NBMPR by 5'-nucleotidase), hydroxynitrobenzylthioguanosine and nitrobenzylthioguanosine, congeners of NBMPR and potent inhibitors of nucleoside transport, were also potent inhibitors of the binding of [^{3}H]NBMPR with K_i values less than 12 nM (Figure 20 & 21, Table 16).

Other inhibitors of nucleoside transport which also inhibited the site-specific binding of [^{3}H]NBMPR to cortical synaptosomes in an apparently competitive manner were (in decreasing order of potency): dilazep, hexodendine, dipyridamole, lidoflazine, and papaverine (Figure 21, Table 16); this is similar to their order of effectiveness for inhibiting the binding of [^{3}H]NBMPR to human erythrocytes (Table 16). Preincubation of the synaptosomes with physostigmine (10 μM), which did not affect the binding of [^{3}H]NBMPR *per se*, more than doubled the

TABLE 15 Maximal specific binding (B_{max}) and apparent dissociation constant (K_D) of [^{3}H]NBMPR in various subcellular fractions of guinea pig cortical membranes.

Membrane Fraction	B_{max} (fmol/mg protein)	K_D (nM)
Homogenate	363 ± 19	0.28 ± 0.03
P_1	355 ± 37	0.31 ± 0.03
P_2	343 ± 16	0.23 ± 0.01
Subfractionation of P_2		
Myelin	310 ± 24	0.29 ± 0.04
Synaptosomes	316 ± 28	0.24 ± 0.02
Mitochondria	185 ± 7	0.24 ± 0.01

Each value represents the mean ± s.e.mean from three experiments performed in duplicate using centrifugation to terminate the binding assays.

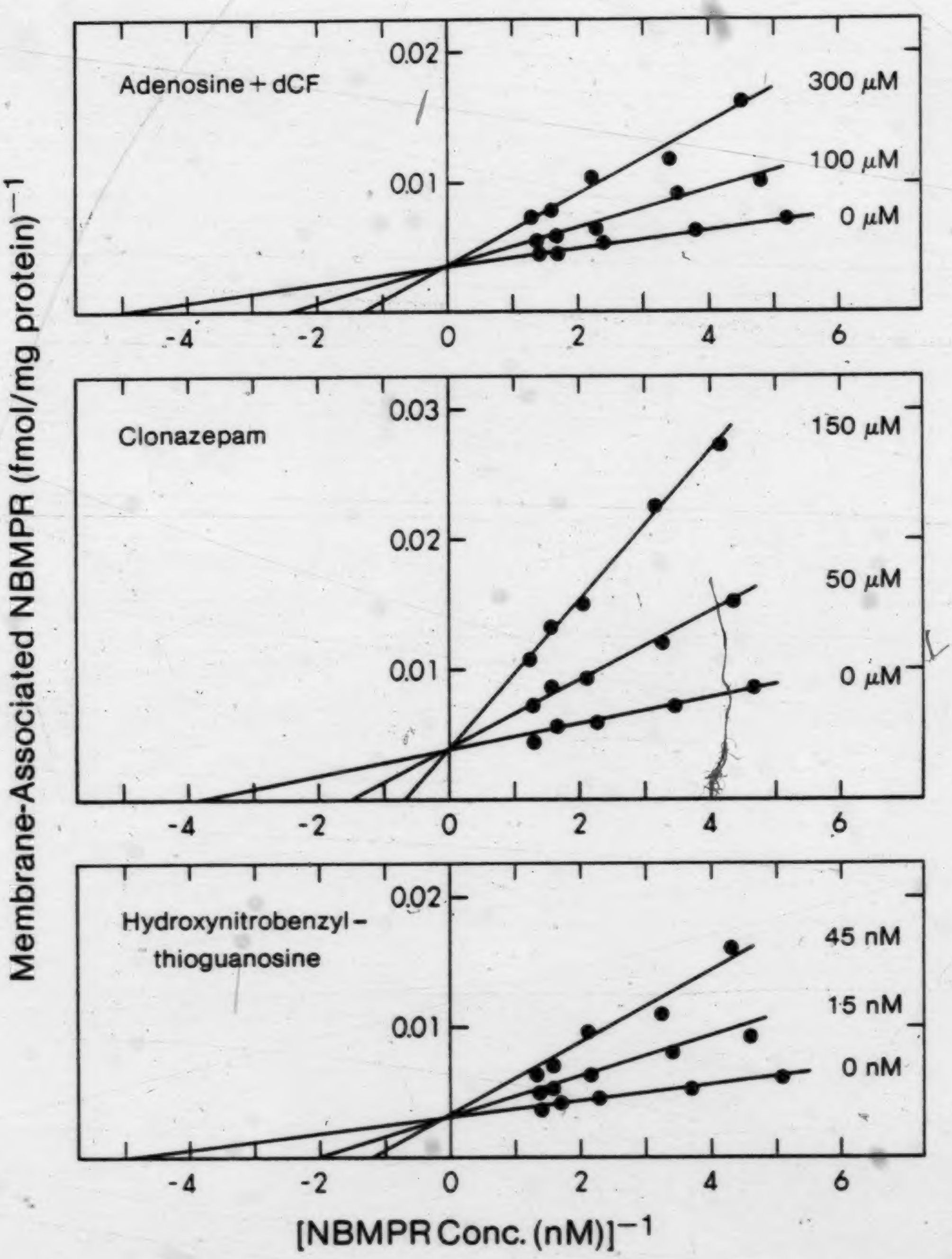


FIGURE 20 Double reciprocal plot analysis of the inhibition of the site-specific binding of [^{3}H]NBMPR to guinea pig cortical membranes, by adenosine in the presence of deoxycoformycin, clonazepam, and hydroxynitrobenzylthioinosine.

TABLE 16 Comparison of the inhibition constants (K_i) of recognized nucleoside transport inhibitors for the inhibition of the site-specific binding of [^{3}H]NBMPR to both guinea pig CNS membranes and human erythrocytes

Inhibitor	Guinea Pig CNS Membranes K_i (nM)	Human Erythrocytes K_i (nM)
Nitrobenzylthioinosine	0.22 ± 0.03	-
Nitrobenzylthioinosine phosphate	0.29 ± 0.01	-
Nitrobenzylthioguanosine	0.36 ± 0.03	1.2*
Dilazep + Physostigmine	0.42 ± 0.03	-
Dilazep - Physostigmine	1.1 ± 0.2	0.29
Hexobendine + Physostigmine	3.2 ± 0.4	-
Hexobendine - Physostigmine	6.7 ± 1.4	1.9
Hydroxynitrobenzylthioguanosine	11 ± 1	11*
Dipyridamole	11 ± 2	1.9
Lidoflazine	100 ± 13	42
Papaverine	5100 ± 800	3500

* These K_i values were calculated from the corresponding IC_{50} values according to the equation $K_i = IC_{50}/[1 + (\text{NBMPR conc}/K_D)]$ where 'NBMPR conc' is the concentration of [^{3}H]NBMPR used for the IC_{50} determination and 'K_D' is the dissociation constant of [^{3}H]NBMPR. All other K_i values were determined directly by double reciprocal plot analysis(see Section F.2.d. of Methods).

Results are the means ± s.e.mean from at least three experiments performed in duplicate.

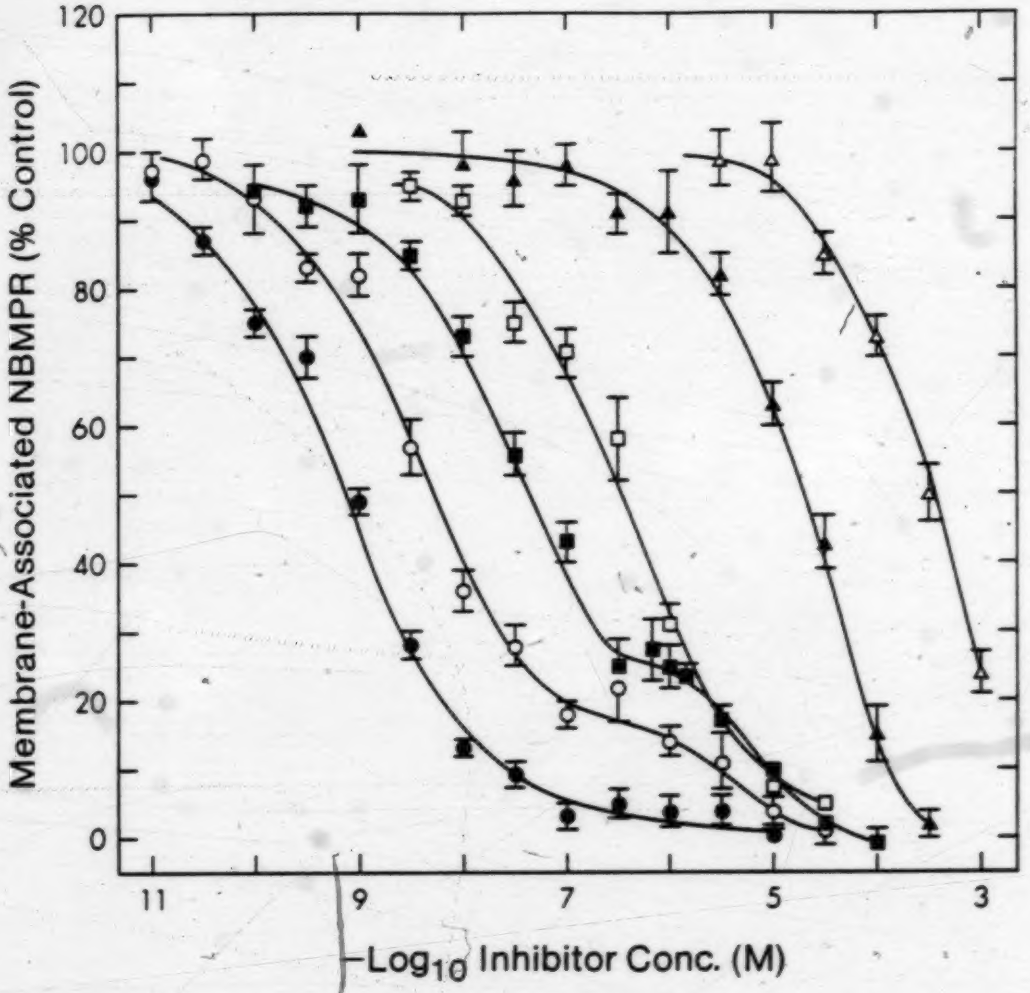


FIGURE 21 Inhibition of the site-specific binding of [^{3}H]NBMPR (0.35 nM, initial concentration) to guinea pig cortical membranes: Concentration-inhibition plots. Inhibitors: nitrobenzylthioguanosine(●); dilazep(○); dipyridamole(■); lidoflazine(□); Ro 5-4864(▲); adenosine(△). Each point represents the mean ± s.e.mean, n = 6.

apparent affinity of dilazep and hexobendine for the [^{3}H]NBMPR site (Table 16).

Several purine and pyrimidine nucleosides which are substrates for the nucleoside transport system were apparently competitive inhibitors of the site-specific binding of [^{3}H]NBMPR (Figure 20). Of the nucleosides tested (Table 17), adenosine was the most potent permeant with a K_i value of 123 μM. Preincubation of cortical synaptosomes with the adenosine deaminase inhibitor, deoxycoformycin, significantly increased the apparent affinity (K_i) of adenosine to 76 μM. 2-Chloroadenosine, a potent agonist at adenosine receptors, was also an effective inhibitor of the binding of [^{3}H]NBMPR with a K_i value of 14 μM (Table 17). Nucleotides, on the other hand, were poor inhibitors of the site-specific binding of [^{3}H]NBMPR; ATP produced 8.3 ± 2.4% and 78 ± 2% inhibition at 1 mM and 10 mM, respectively. Similarly, GTP produced a 19 ± 3% and 49 ± 10% inhibition at 1 mM and 3 mM, respectively.

Benzodiazepines inhibited the site-specific binding of [^{3}H]NBMPR to guinea pig cortical synaptosomes with potencies similar to those determined for the inhibition of the binding of [^{3}H]NBMPR to human erythrocytes (Table 18). Ro 5-4864, midazolam, diazepam, clonazepam, and lorazepam were apparently competitive inhibitors, with K_i values ranging from 5.4 μM for Ro 5-4864 to 52 μM for lorazepam (Figure 20 & 21, Table 18). Flurazepam and its active metabolite, didesethylflurazepam, were very weak inhibitors of site-specific binding of [^{3}H]NBMPR, as was the potent benzodiazepine antagonist, Ro 15-1788; each produced approximately 50% inhibition at a concentration of 300 μM (Table 19). The interaction of the benzodiazepines with the [^{3}H]NBMPR binding site in guinea pig cortical synaptosomes, as in human erythrocytes, appears to

TABLE 17 Comparison of the inhibition constants (K_i) of nucleosides for the inhibition of the site-specific binding of [^{3}H]NBMPR to guinea pig CNS membranes

Inhibitor	K_i (μM)
2-Chloroadenosine	14 ± 1
Adenosine + deoxycoformycin	76 ± 6
Adenosine - deoxycoformycin	123 ± 17
Inosine	325 ± 32
Thymidine	398 ± 68
Guanosine	425 ± 32
Uridine	940 ± 165
Cytidine	3000 ± 300

Results are the means ± s.e.mean from at least three experiments performed in duplicate. All K_i values were determined by double reciprocal plot analysis.

TABLE 18 Comparison of the inhibition constants (K_i) of benzodiazepines for the inhibition of the site-specific binding of [^{3}H]NBMPR to both guinea pig CNS membranes and human erythrocytes

Inhibitor	Guinea Pig CNS Membranes K_i (μM)	Human Erythrocytes K_i (μM)
Ro 5-4864	5.4 ± 0.6	2.2
Ro 11-6893(-)	6.9 ± 0.5	18*
Midazolam	8.5 ± 0.5	24*
Diazepam	16 ± 2	6.8
Clonazepam	34 ± 5	24
Ro 11-6896(+)	41 ± 3	130*
Lorazepam	52 ± 14	45

* See Table 16 for explanation.

Results are the means ± s.e.mean from at least three experiments performed in duplicate.

TABLE 19 Inhibition of the site-specific binding of [^{3}H]NBMPR to guinea pig CNS membranes by miscellaneous compounds

Compound	% Inhibition at 300 µM*
Benzodiazepines	
Flurazepam	56 ± 3
Didesethylflurazepam	50 ± 2
Ro 15-1788	47 ± 2
β-Carbolines	
Ethyl β-carboline-3-carboxylate(βCCE)	76 ± 3
Ethyl β-carboline-3-carboxamide	62 ± 2
(-) Ethyl 1,2,3,4-tetrahydro-β-carboline-3-carboxylate	55 ± 3
Phenothiazines	
Fluphenazine	66 ± 3
Thiothixene-*cis*	64 ± 2
Trifluoperazine	62 ± 2
Thiothixene-*trans*	56 ± 3
Thioridazine	37 ± 2
Chlorpromazine	36 ± 5
Corticosterone	58 ± 6
Hydrocortisone	53 ± 5
Indomethacin	43 ± 3
Physostigmine	41 ± 1

TABLE 19 continued

Compound	% Inhibition at 300 μM*
Haloperidol	36 ± 6
Phenytoin	32 ± 4
Morphine	20 ± 6
Thiopental	20 ± 3

* % Inhibition values were determined using 0.35 nM [^{3}H]NBMPR. Results are the means ± s.e.mean from three experiments performed in duplicate.

be influenced by the conformation at one benzodiazepine chiral center because the (-) isomer, Ro 11-6893, had approximately a 6-fold higher affinity for the site than the (+) benzodiazepine isomer, Ro 11-6896 (Table 18). The site-specific binding of $[^3H]$NBMPR was not influenced by benzodiazepines at concentrations of less than 300 nM (Figure 21).

Hill coefficients calculated from the data shown in Figure 21 for the inhibition of the binding of $[^3H]$NBMPR by adenosine, Ro 5-4864, and diazepam indicated a non-cooperative type of interaction (n_H values greater than 0.75). However, lidoflazine, dipyridamole, dilazep, and nitrobenzylthioguanosine had Hill coefficients significantly less than 0.75 (Table 20). Dipyridamole and dilazep also displayed biphasic competition curves (Figure 21) with a plateau occuring at inhibitor concentrations of 0.3 μM to 1.0 μM.

Phenothiazines and ethyl β-carboline-3-carboxylate (βCCE) and cogeners were weak inhibitors of the binding of $[^3H]$NBMPR (Table 19). In addition, a wide variety of other compounds were tested for their abilities to inhibit the binding of $[^3H]$NBMPR (Tables 19 & 21). Of these, only corticosterone, hydrocortisone, indomethacin, haloperidol, phenytoin, morphine, physostigmine, and thiopental produced a significant inhibition at a concentration of 300 μM (Table 19).

4) Rate of dissociation of $[^3H]$NBMPR from CNS membranes: The rate of dissociation of $[^3H]$NBMPR from its specific sites in cortical membranes (Figure 22) was measured, using a filtration procedure, in the presence of nonradioactive 'displacers' (dipyridamole, 100 μM; nitrobenzylthioinosine phosphate, 12 μM; diazepam, 625 μM; adenosine, 8 mM). These inhibitor concentrations are at least 100-fold higher than their respective K_i values for inhibition of the site-specific binding of

TABLE 20 Inhibition of the site-specific binding of [^{3}H]NBMPR to guinea pig CNS membranes: IC_{50} values and Hill coefficients (n_H values) for various inhibitors

Inhibitor	IC_{50}*	n_H
	(nM)	
Nitrobenzylthioguanosine	0.93 ± 0.06	0.64 ± 0.01
Dilazep	4.6 ± 0.1	0.65 ± 0.03
Dipyridamole	48 ± 8	0.52 ± 0.02
Lidoflazine	360 ± 73	0.71 ± 0.01
	(μM)	
Ro 5-4864	21 ± 4	0.97 ± 0.06
Diazepam	43 ± 2	0.77 ± 0.03
Adenosine	266 ± 47	0.88 ± 0.02

* IC_{50} values were determined using 0.35 nM [^{3}H]NBMPR. Results are the means ± s.e.mean from at least three experiments performed in duplicate.

TABLE 21 Agents which do not inhibit the site-specific binding of [^{3}H]NBMPR to guinea pig CNS membranes or human erythrocytes

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Inactive at 300 µM

Guinea Pig CNS Membranes:

Acetylsalicylic acid, cimetidine, cocaine, deoxycoformycin, adrenaline, fentanyl, histamine, desimipramine, meperidine, ouabain, pancuronium, phentolamine, propranolol, theophylline, tubocurarine.

Human Erythrocytes:

Acetylsalicylic acid, cimetidine, cocaine, fentanyl, histamine, hydrocortisone, imipramine, ketamine, mepyramine, morphine, ouabain, phentolamine, propranolol, theophylline, thiopental, tubocurarine.

Inactive at 10 mM

Guinea Pig CNS Membranes:

Acetylcholine, dopamine, *gamma*-aminobutyric acid, glucose, serotonin.

Human Erythrocytes:

Adrenaline, *gamma*-aminobutyric acid, glucose, glycine, proline tryptophan.

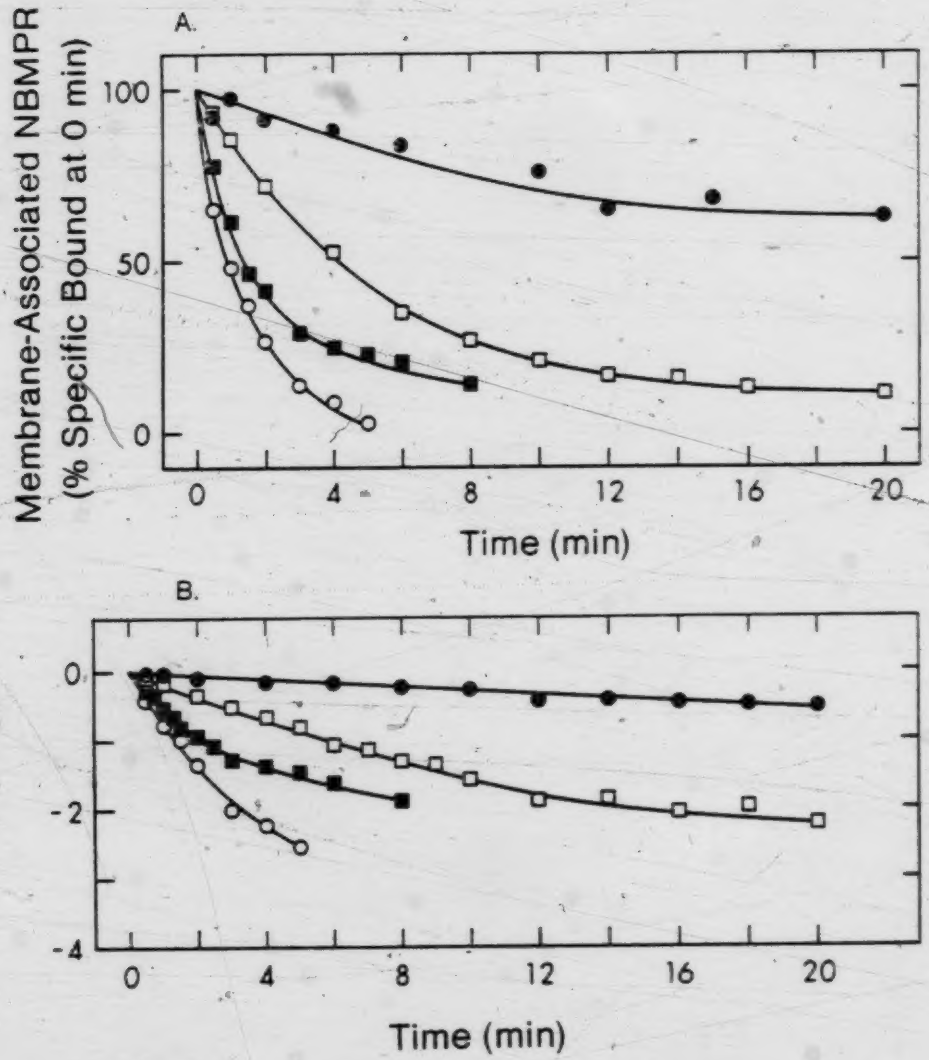

FIGURE 22 Dissociation of site-bound [^{3}H]NBMPR from guinea pig cortical membranes at 22°C. Dissociation was initiated using either 100 μM dipyridamole(●), 12 μM nitrobenzylthioinosine phosphate(□), 625 μM diazepam(■) or 8 mM adenosine(○) as the 'displacer'. Data were plotted on an arithmetic scale(A) and on a semi-logarithmic scale(B). Dissociation rate constants(k_2) may be calculated as the slopes of the lines in B. Each point is the mean, n = 4.

[^{3}H]NBMPR and are sufficient to displace all site-bound [^{3}H]NBMPR. A semilogarithmic plot (Figure 22B) of the site-bound [^{3}H]NBMPR remaining at various times after the addition of 'displacer' indicated that the dissociation of [^{3}H]NBMPR from its sites was biphasic and the rates varied depending on the 'displacer' used. The use of dipyridamole as the 'displacer' resulted in the slowest rate of dissociation ($t_{1/2}$ = 24 min) followed by nitrobenzylthioinosine phosphate and then diazepam (Table 22). Adenosine produced the most rapid rate of dissociation of [^{3}H]NBMPR, 28 times faster than that produced by dipyridamole (Table 22). The equilibrium dissociation constant for [^{3}H]NBMPR, obtained as the ratio of k_2/k_1 (where k_1 = 0.56 min^{-1}, derived from association experiments, and k_2 is the rate of the 'fast' dissociating component), was 0.30 nM when dissociation was initiated by nitrobenzylthioinosine phosphate. Apparent K_D values obtained when dissociation was initiated by dipyridamole, diazepam, or adenosine were 0.04 nM, 0.86 nM, and 1.05 nM, respectively. The K_D value of 0.30 nM determined using nitrobenzylthioinosine phosphate to obtain the dissociation constant (k_2) is similar to the K_D for the binding of [^{3}H]NBMPR determined by mass law analysis (0.25 nM).

5) Regional distribution of [^{3}H]NBMPR binding sites in guinea pig brain: Guinea pig brain exhibited a marked regional heterogeneity in the density of membrane binding sites of [^{3}H]NBMPR. The highest density of sites was found in lower brain areas such as the pons/medulla and lower brain stem. Membranes from hippocampus had the lowest density of these sites and cortex was intermediate in this respect (Table 23, Figure 23). In each brain region examined, [^{3}H]NBMPR bound, in an apparently non-cooperative manner, to a single class of sites, and

TABLE 22 Rate constants for the dissociation of site-bound $[^3H]$NBMPR induced by various 'displacers' in guinea pig CNS membranes

'Displacer'	Dissociation rate constant(k_2) (min^{-1})
Dipyridamole(100 μM)	0.021 ± 0.001
Nitrobenzylthioinosine phosphate(12 μM)	[*]a) 0.167 ± 0.005 [†]b) 0.055 ± 0.012
Diazepam(625 μM)	[*]a) 0.481 ± 0.021 [†]b) 0.093 ± 0.002
Adenosine(8 mM)	0.588 ± 0.044

[*] This is the 'fast' dissociation rate of a biphasic dissociation plot.

[†] This is the 'slow' dissociation rate of a biphasic dissociation plot.

Results are the means ± s.e.mean of four experiments.

TABLE 23 Binding constants for the site-specific binding of [^{3}H]NBMPR to membranes prepared from various regions of guinea pig brain

Brain region	B_{max} (fmol/mg protein)	K_D (nM)	n_H
Pons/Medulla	644 ± 12	0.38 ± 0.04	0.98 ± 0.02
Lower brain stem	623 ± 22	0.34 ± 0.02	0.95 ± 0.02
Thalamus/Hypothalamus	440 ± 6	0.28 ± 0.01	1.04 ± 0.02
Cortex	433 ± 15	0.31 ± 0.02	1.01 ± 0.03
Olfactory lobe	353 ± 14	0.15 ± 0.02	1.08 ± 0.04
Caudate nucleus	310 ± 7	0.22 ± 0.02	0.96 ± 0.02
Cerebellum	299 ± 18	0.31 ± 0.03	1.01 ± 0.03
Hippocampus	201 ± 10	0.20 ± 0.02	1.00 ± 0.07

Each experiment was performed using pooled brain regions from 5 or 6 guinea pigs.

Results are the means ± s.e.mean from three experiments conducted in duplicate.

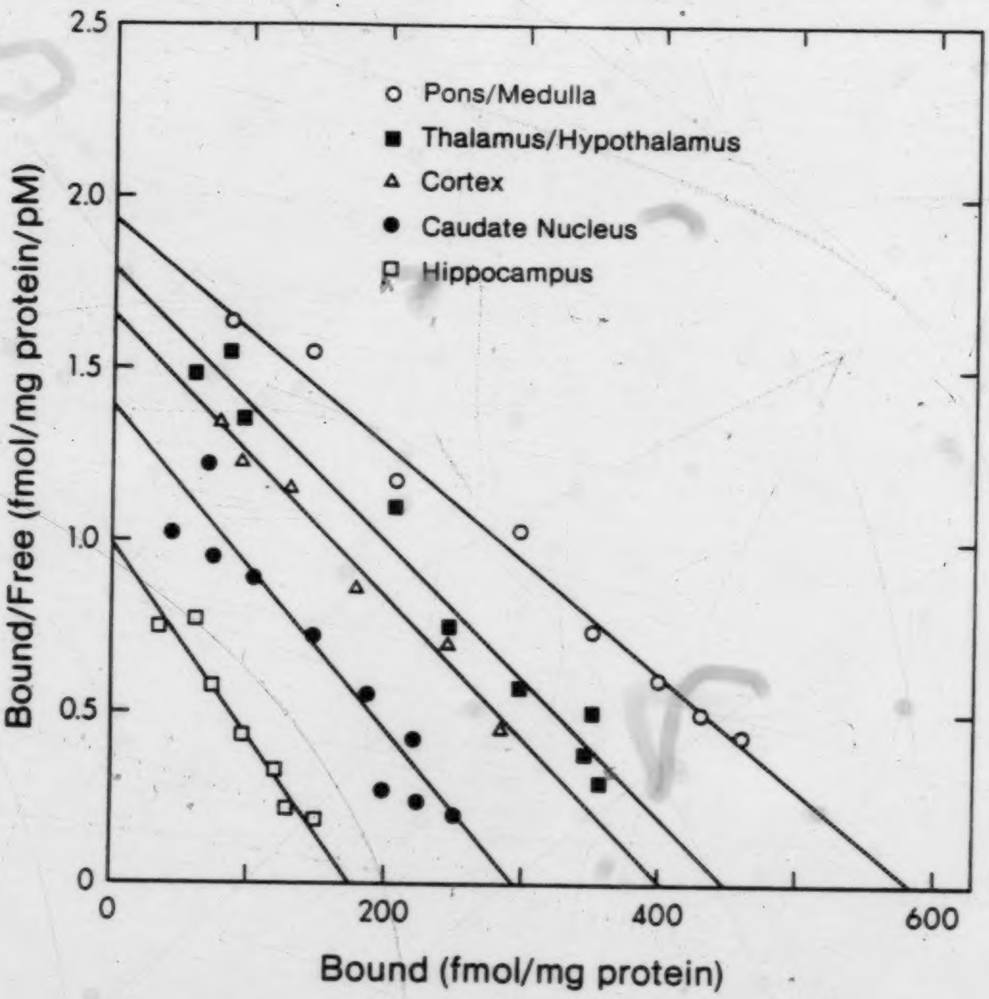

FIGURE 23 Regional differences in the site-specific binding of $[^3H]$NBMPR to guinea pig brain membranes. These are representative Scatchard plots which are not different from those obtained from 5 other experiments of a similar type.

dissociation constants for [^{3}H]NBMPR at these sites, ranged from 0.15 nM in the olfactory lobe to 0.38 nM in the pons/medulla. There were no marked regional variations in the affinity of dipyridamole or diazepam for the [^{3}H]NBMPR site. These agents competitively inhibited the binding of [^{3}H]NBMPR to membranes prepared from areas of high (pons/medulla) and low (hippocampus) binding site densities; observed were K_i values of 6 nM and 7 nM respectively, for dipyridamole, and 13 μM and 21 μM respectively, for diazepam.

D. Species Differences in the Binding of [^{3}H]NBMPR to CNS Membranes

1) [^{3}H]NBMPR binding constants: Scatchard analysis of the site-specific component of the binding of [^{3}H]NBMPR indicated an apparent single class of binding sites for [^{3}H]NBMPR in rat, mouse, dog, and guinea pig cortical membranes (Figure 24). The affinity of these sites for [^{3}H]NBMPR ranged from 0.11 nM in rat and mouse cortex to 4.9 nM in dog cortex. [^{3}H]NBMPR binding site density also varied widely among those species (Table 24). Hill coefficients calculated from these data were greater than 0.75 in each species indicating that the interaction of [^{3}H]NBMPR with its sites was apparently non-cooperative (Table 24). Analysis of the site-specific binding of [^{3}H]NBMPR to rabbit cortical membranes yielded non-linear Scatchard plots (Figure 24B) which were resolved into two linear components. This indicated the possible existence of two distinct binding sites for [^{3}H]NBMPR in rabbit cortical membranes with K_D values of 4.1 nM and 0.32 nM (Table 24).

2) Inhibition of the binding of [^{3}H]NBMPR: Diazepam inhibited, in a concentration-dependent manner, the site-specific binding of [^{3}H]NBMPR

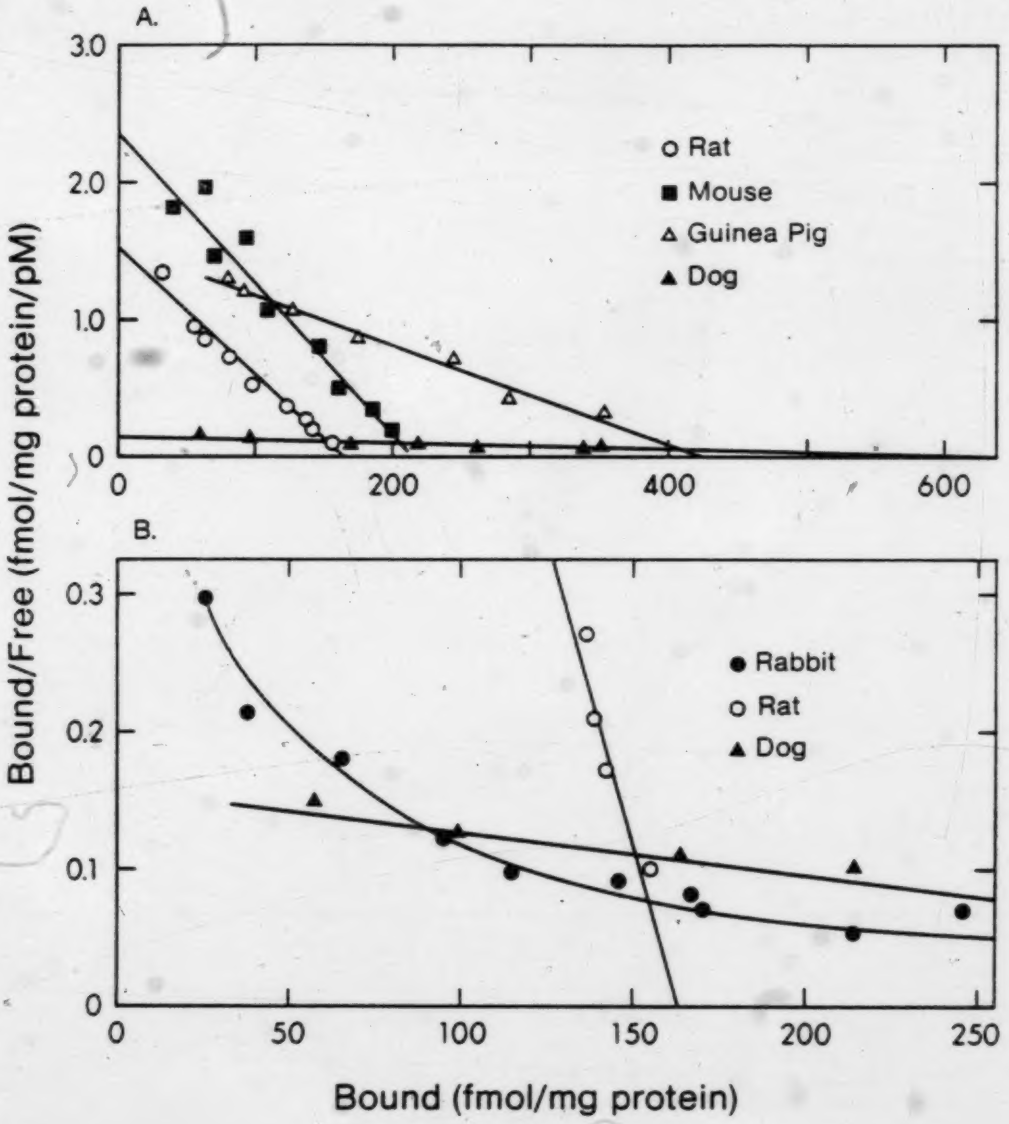

FIGURE 24 Site-specific binding of $[^3H]$NBMPR to rat, mouse, guinea pig, dog and rabbit cortical membranes: Scatchard plots. These are representative plots which are not different from those obtained from at least 5 other experiments of a similar type.

TABLE 24 Species differences in the site-specific binding of [^{3}H]NBMPR to mammalian cortical membranes

Species	B_{max} (fmol/mg protein)	K_D (nM)	n_H
Rat(12)	172 ± 9	0.11 ± 0.01	1.03 ± 0.02
Mouse(6)	210 ± 14	0.11 ± 0.02	0.95 ± 0.02
Guinea pig(6)	433 ± 15	0.31 ± 0.02	1.01 ± 0.03
Dog(8)	638 ± 44	4.9 ± 0.5	1.08 ± 0.03
Rabbit(6)	*a) 79 ± 11	a) 0.32 ± 0.04	†0.83 ± 0.03
	b) 520 ± 98	b) 4.1 ± 0.6	

* Rabbit cortical membranes contained two distinct binding sites for [^{3}H]NBMPR which are represented in the table as a) and b) for the 'high' affinity and 'low' affinity sites, respectively.

† Hill coefficients(n_H values) were calculated for the binding of [^{3}H]NBMPR to rabbit cortical membranes using data obtained for all concentrations of [^{3}H]NBMPR.

Results are the means ± s.e.mean from the number of experiments indicated in parentheses.

to cortical membranes prepared from each species with IC_{50} values ranging from 8.8 μM to 82 μM depending upon the species and the initial concentration of $[^3H]$NBMPR used in each assay (Table 25, Figure 25 & 26). Hill coefficients calculated for diazepam inhibition of the binding of $[^3H]$NBMPR were not different from unity (n_H values greater than 0.75) in rat, mouse, and guinea pig cortical membranes indicating a non-cooperative type of site interaction. On the other hand, in dog and rabbit cortical membranes, Hill coefficients of less than 0.75 were obtained for the inhibition of the binding of $[^3H]$NBMPR by diazepam which suggests either negative cooperativity or binding site heterogeneity (Table 25). Furthermore, in these membranes, increasing the concentration of $[^3H]$NBMPR in the assays resulted in a parallel shift of the diazepam concentration-inhibition plots to the right (Figure 26), which indicates an apparently competitive type of inhibition.

Several benzodiazepines including diazepam, Ro 5-4864, clonazepam, and the benzodiazepine stereoisomer pair, Ro 11-6893(-) and Ro 11-6896 (+), inhibited the binding of $[^3H]$NBMPR to rat cortical membranes in an apparently competitive manner (as determined by double reciprocal plot analysis) with K_i values similar to those determined for the inhibition of the binding of $[^3H]$NBMPR to guinea pig cortical membranes (Table 26).

Dipyridamole, in contrast to diazepam, displayed a biphasic inhibition plot for the inhibition of the binding of $[^3H]$NBMPR to rabbit, dog, and guinea pig cortical membranes (Figure 25 & 26). For instance, in rabbit cortical membranes, using an initial concentration of 2.0 nM $[^3H]$NBMPR to label the sites, 60% of the binding was inhibited by 100 nM dipyridamole, whereas 100 μM dipyridamole was required to inhibit the remaining binding component (40%). IC_{50} values calculated for each

TABLE 25 Species differences in the inhibition of the site-specific binding of $[^3H]$NBMPR to mammalian cortical membranes by diazepam

Species	$[^3H]$NBMPR* (nM)	IC_{50} (μM)	n_H
Rat	0.15	30 ± 6	0.93 ± 0.07
Mouse	0.15	43 ± 5	0.99 ± 0.10
Guinea Pig	0.35	43 ± 2	0.77 ± 0.03
Rabbit	0.35	8.8 ± 1.1	0.80 ± 0.05
	2.0	25 ± 4	0.53 ± 0.01
Dog	0.35	16 ± 5	0.63 ± 0.03
	4.0	82 ± 16	0.73 ± 0.09

* The initial concentration of $[^3H]$NBMPR used in the inhibition assays.

Results are the means ± s.e.mean from three experiments performed in duplicate.

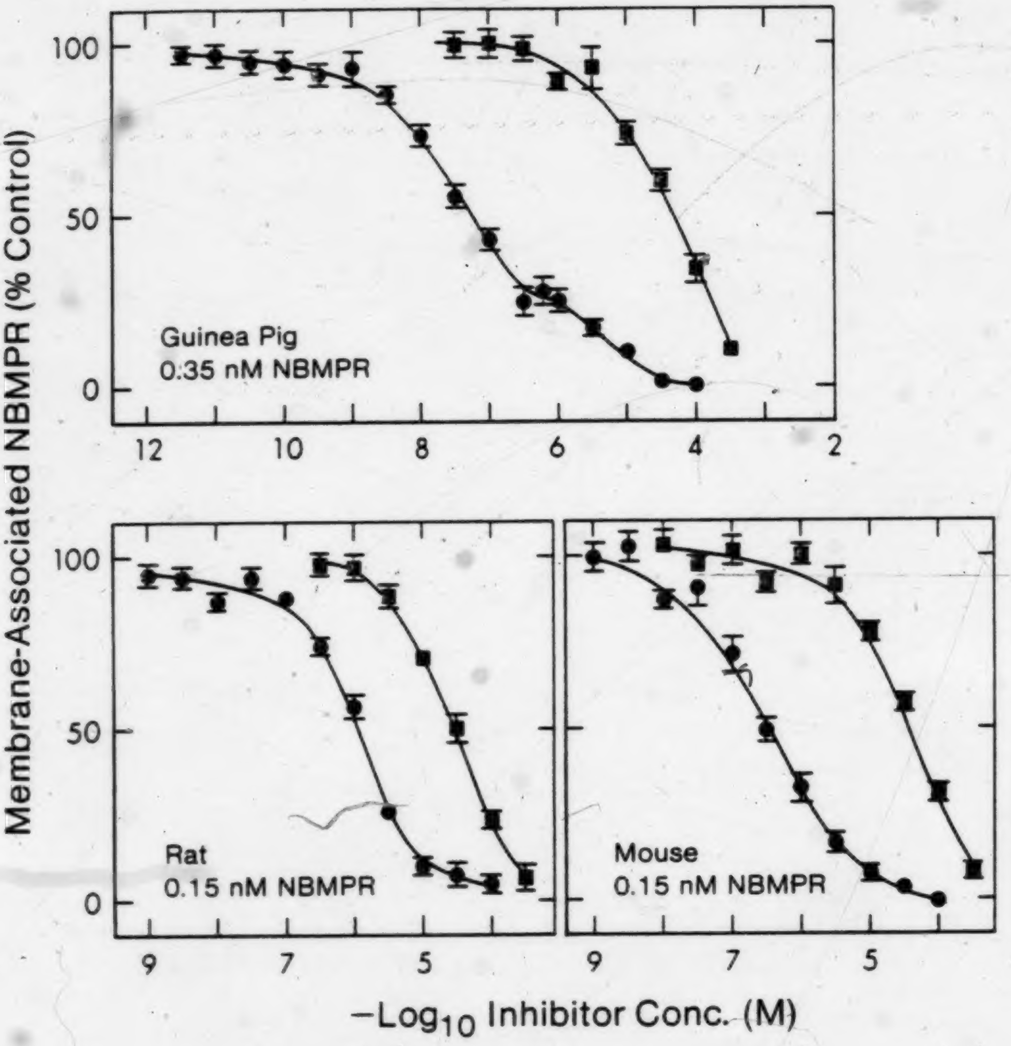

FIGURE 25 Species differences in the inhibition of the site-specific binding of $[^3H]$NBMPR to mammalian cortical membranes by diazepam and dipyridamole - I. Guinea pig, rat and mouse cortical membranes were incubated with $[^3H]$NBMPR(initial concentration as indicated) in the presence and absence of a range of concentrations of diazepam(■) or dipyridamole(●). Each point represents mean ± s.e.mean, n = 6.

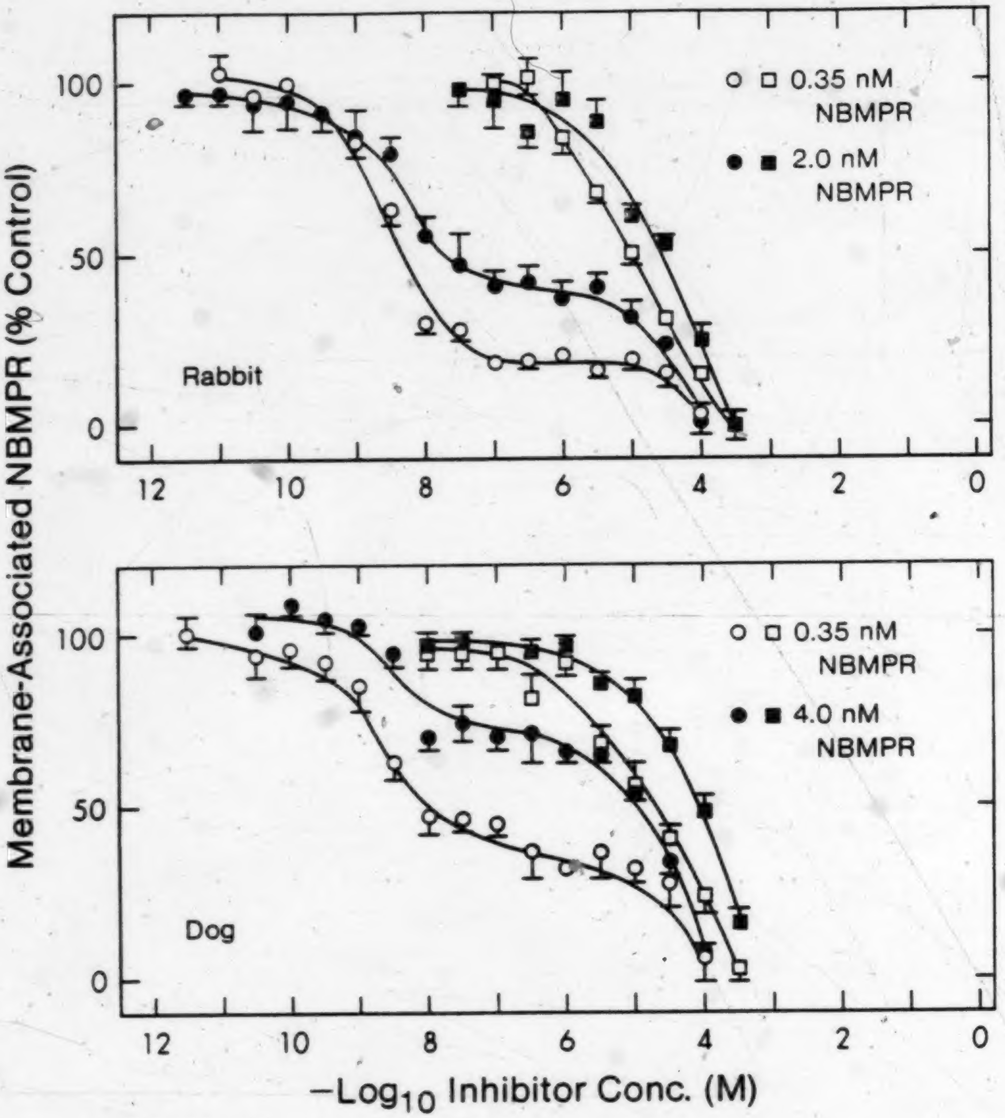

FIGURE 26 Species differences in the inhibition of the site-specific binding of [^{3}H]NBMPR to mammalian cortical membranes by diazepam and dipyridamole - II. Rabbit and dog cortical membranes were incubated with [^{3}H]NBMPR(initial concentration as indicated) in the presence and absence of a range of concentrations of diazepam(□■) or dipyridamole(○●). Each point represents mean ± s.e.mean, n = 6.

TABLE 26 Comparison of the inhibition constants(K_i) of dipyridamole and several benzodiazepines for the inhibition of the site-specific binding of [^{3}H]NBMPR to rat and guinea pig cortical membranes

Inhibitor	Rat Cortex K_i (μM)	Guinea Pig Cortex K_i (μM)
Dipyridamole	0.404 ± 0.044	0.011 ± 0.02
Ro 5-4864	5.1 ± 0.8	5.4 ± 0.6
Diazepam	14 ± 2	16 ± 2
Ro 11-6893(-)	20 ± 3	6.9 ± 0.5
Clonazepam	46 ± 5	34 ± 5
Ro 11-6896(+)	64 ± 13	41 ± 3

Results are the means ± s.e.mean from three experiments performed in duplicate.

phase are shown in Table 27. In these rabbit membranes, the ratio of $[^3H]$NBMPR sites displaying 'high' and 'low' affinity for dipyridamole is approximately 60/40. It is of interest to note that this ratio is the same as that obtained for the sites with 'high' and 'low' affinity for 2.0 nM $[^3H]$NBMPR (i.e., 200 fmol of 2.0 nM $[^3H]$NBMPR was bound/mg protein, in total, of which 105 fmol/mg were bound to 'high' affinity $[^3H]$NBMPR sites, calculated from data shown in Figure 24). If a relatively lower concentration of $[^3H]$NBMPR was used in the inhibition assays (e.g., 0.35 nM $[^3H]$NBMPR with rabbit cortical membranes), thereby decreasing the number of 'low' affinity (Figure 24) $[^3H]$NBMPR sites labelled, the proportion of sites displaying 'high' affinity for dipyridamole increased (Figure 26, Table 27). This again suggests that the sites displaying 'high' affinity for dipyridamole correspond to the sites displaying 'high' affinity for $[^3H]$NBMPR in rabbit cortical membranes. A similar relationship between $[^3H]$NBMPR concentration and the ratio of sites displaying 'high' and 'low' affinity for dipyridamole was observed using dog cortical membranes (Figure 26, Table 27) although, in these membranes, Scatchard analysis had indicated that $[^3H]$NBMPR bound to an apparent single class of sites (Figure 24). Dipyridamole displayed approximately a 10000-fold lower affinity for 'low' affinity $[^3H]$NBMPR sites (IC_{50} = 40 μM) than for 'high' affinity $[^3H]$NBMPR sites (IC_{50} = 4 nM) in rabbit and dog cortical membranes (Table 27). In guinea pig cortical membranes, however, the affinities of these sites for dipyridamole only differed by approximately 300-fold (Table 27). In contrast, all of the specific binding sites for $[^3H]$NBMPR in rat cortical membranes displayed a low affinity for dipyridamole (K_i = 0.404 μM); a similar, apparently homogeneous, population of sites exist in

TABLE 27 Species differences in the inhibition of the site-specific binding of $[^3H]$NBMPR to mammalian cortical membranes by dipyridamole

Species	$[^3H]$NBMPR[*] (nM)	IC_{50} Site A[†] (nM)	IC_{50} Site B[†] (μM)	A/B[¶]	n_H[§]
Rat	0.15	-	1.23 ± 0.07	0/100	0.80 ± 0.11
Mouse	0.15	-	0.36 ± 0.07	0/100	0.80 ± 0.05
Guinea Pig	0.35	20 ± 3	6.3 ± 1.1	75/25	-
Rabbit	0.35	3.8 ± 0.9	31 ± 5	82/18	-
	2.0	4.2 ± 0.8	48 ± 12	60/40	-
Dog	0.35	2.2 ± 0.4	38 ± 4	65/35	-
	4.0	7.5 ± 1.6	45 ± 8	32/68	-

[*] The concentration of $[^3H]$NBMPR used in the inhibition assays.

[†] In guinea pig, rabbit, and dog cortical membranes, dipyridamole exhibited biphasic inhibition plots. 'Site A' and 'Site B' represent the sites which display high and low affinity for dipyridamole, respectively.

[¶] This value represents the ratio of $[^3H]$NBMPR sites displaying high and low affinity for dipyridamole when using the indicated concentration of $[^3H]$NBMPR.

[§] Hill coefficients(n_H) values were calculated using data only from monophasic dipyridamole inhibition plots.

Results are the means ± s.e.mean from three experiments performed in duplicate.

mouse cortical membranes which display an apparent K_i value for dipyridamole of 0.152 μM (Table 27, Figure 25).

3) Dissociation rates of [^{3}H]NBMPR: In each species tested (rat, guinea pig, rabbit) the rate of dissociation of [^{3}H]NBMPR from its sites in cortical membranes was dependent upon the 'displacer' used (Figure 27). Dipyridamole induced the slowest dissociation rate of [^{3}H]NBMPR in each species (Figure 27, Table 28). It was an extremely weak displacer of [^{3}H]NBMPR from the sites in rat cortical membranes (k_2 = 0.002 min^{-1}) even though the dipyridamole concentration used (100 μM) was sufficient to inhibit all the site-specific binding of [^{3}H]NBMPR to rat cortical membranes in competition experiments. In each species, nitrobenzylthioinosine phosphate induced a 3- to 15-fold faster rate of dissociation of [^{3}H]NBMPR from its sites than did dipyridamole (Table 28, Figure 27). Adenosine induced the fastest rate of dissociation of [^{3}H]NBMPR from its sites in rat and guinea pig cortical membranes, approximately 1.2 times and 40 times faster than that induced by diazepam and dipyridamole, respectively, On the other hand diazepam produced the fastest rate of dissociation of site-bound [^{3}H]NBMPR in rabbit cortical membranes (Table 28, Figure 27). The rate of dissociation of [^{3}H]NBMPR from rat cortical membranes was monophasic with all 'displacers'; this is in contrast to data derived from a) guinea pig cortical membranes where nitrobenzylthioinosine phosphate or diazepam induced biphasic rates of dissociation of [^{3}H]NBMPR, and b) rabbit cortical membranes where nitrobenzylthioinosine phosphate, diazepam, adenosine, and dipyridamole induced biphasic rates of dissociation of [^{3}H]NBMPR (Figure 27). In general, the rates of dissociation of [^{3}H]NBMPR from its sites in rat cortical membranes were about

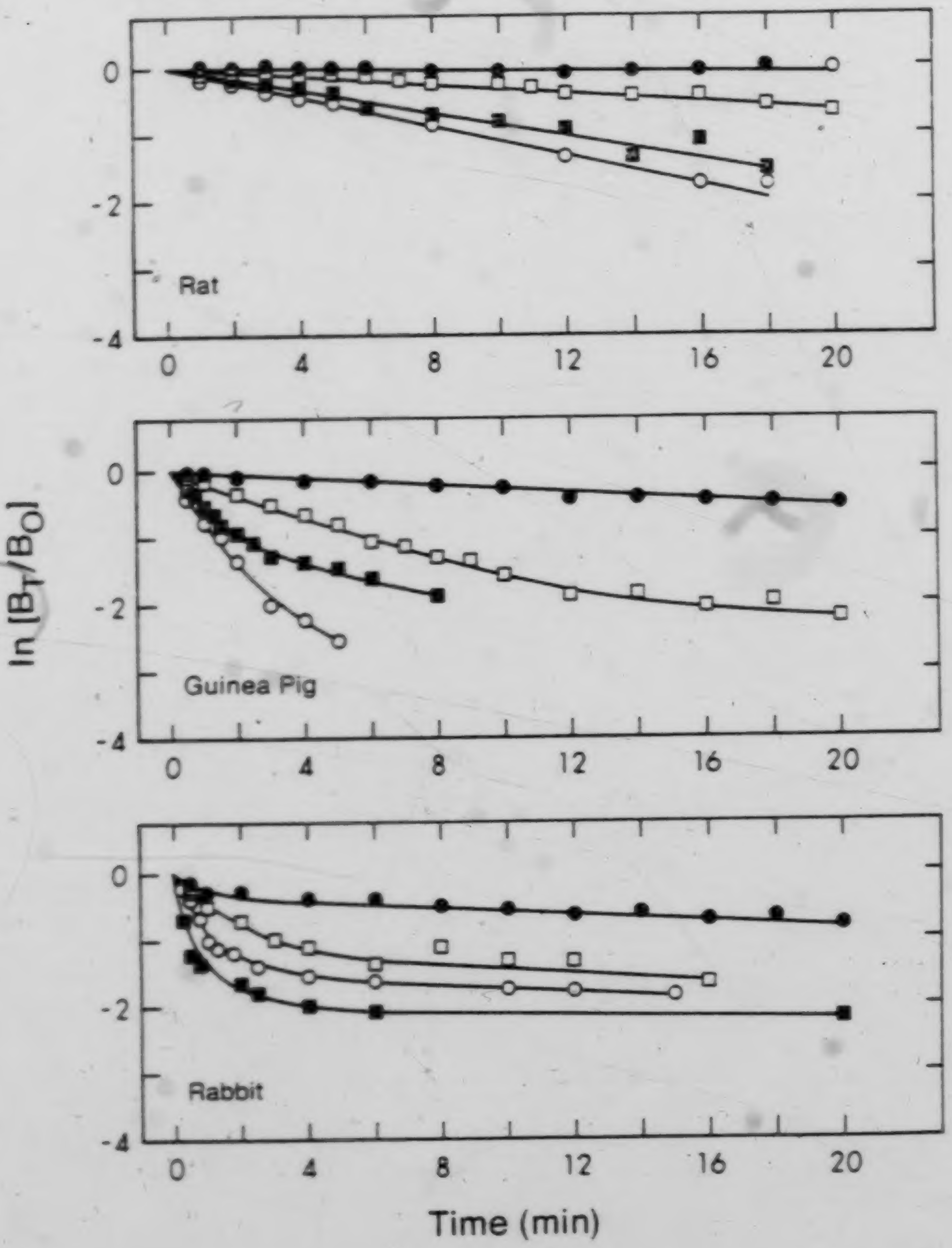

FIGURE 27 Species differences in the rate of dissociation of site-bound [^{3}H]NBMPR from mammalian cortical membranes at 22°C. 'Displacers': dipyridamole(●), nitrobenzylthioinosine phosphate(□), diazepam(■), adenosine(○). Each point is the mean of 4 experiments.

TABLE 28 Species differences in the rate of dissociation of $[^3H]$NBMPR from its sites in mammalian cortical membranes

'Displacer'		Dissociation Rate Constant-k_2 (min^{-1})		
		Guinea Pig	Rabbit	Rat
Dipyridamole	[*] a)	-	0.166 ± 0.024	-
	b)	0.021 ± 0.001[†]	0.019 ± 0.004	0.002 ± 0.001
Nitrobenzylthioinosine phosphate	a)	0.167 ± 0.005	0.526 ± 0.047	-
	b)	0.055 ± 0.012	0.047 ± 0.005	0.030 ± 0.002
Diazepam	a)	0.481 ± 0.021	2.33 ± 0.18	-
	b)	0.093 ± 0.002	0.056 ± 0.011	0.077 ± 0.006
Adenosine	a)	0.588 ± 0.044	0.989 ± 0.137	-
	b)	-	0.041 ± 0.011	0.100 ± 0.003

[*] a) and b) represent the 'fast' and 'slow' dissociation rates, respectively, of biphasic $[^3H]$NBMPR dissociation plots.

[†] The reporting of only one dissociation rate constant indicates that the dissociation of $[^3H]$NBMPR from its sites was monophasic in this case. The classification of this rate as a) or b) depended on whether the rate most closely resembled the 'fast' or 'slow' rates, respectively, of biphasic dissociation plots.

Results are the means ± s.e.mean from four experiments.

5 times slower than the corresponding 'fast' dissociation rates of [^{3}H]NBMPR from its sites in guinea pig cortical membranes. For individual 'displacers', the most rapid rate of [^{3}H]NBMPR dissociation was observed when using rabbit cortical membranes (Table 28).

The rates of dissociation of [^{3}H]NBMPR during the 'slow' dissociation phase in both rabbit and guinea pig cortical membranes were similar ($k_2 \approx 0.05$ min^{-1}). In contrast to the 'fast' dissociation rates, the 'slow' rates were relatively independent of the 'displacer' used (Table 28). However, the amount of [^{3}H]NBMPR remaining bound following completion of the 'fast' dissociation phase was dependent on the 'displacer' used and, in rabbit membranes in particular, the conversion between the 'fast' and 'slow' dissociation rates occurred at approximately the same time (≈ 2 min) after the addition of each 'displacer' (Figure 27). The association rates (k_1) for the binding of [^{3}H]NBMPR to its two classes of sites in rabbit cortical membranes, calculated as k_2/K_D using nitrobenzylthioinosine phosphate to obtain k_2, were similar; these rates were 0.128 min^{-1} and 0.147 min^{-1} for the 'low' affinity and 'high' affinity [^{3}H]NBMPR sites, respectively.

E. Inhibition of Adenosine Deaminase

Deoxycoformycin inhibited adenosine deaminase (from calf intestinal mucosa) activity in a concentration-dependent manner with an IC_{50} value of 5.8 ± 2.1 nM(4) (Figure 28). Of the other compounds tested (Table 29), dipyridamole, although significantly less potent than deoxycoformycin, was the most potent inhibitor of adenosine deaminase (24% inhibition at 100 μM, final concentration). Benzodiazepines were also very weak inhibitors of adenosine deaminase producing less than 20%

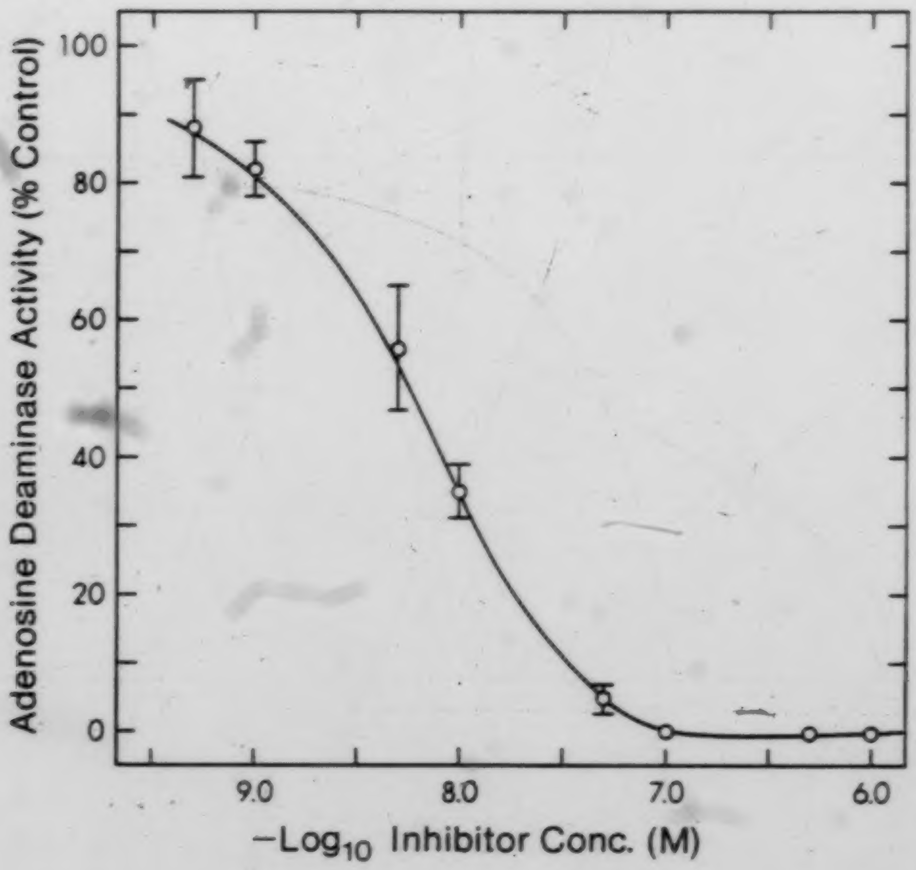

FIGURE 28 Inhibition of adenosine deaminase by deoxycoformycin. Each point represents mean ± s.e.mean, n = 4. The IC_{50} value for inhibition of adenosine deaminase by deoxycoformycin, calculated from these data, is 5.8 nM.

TABLE 29 Inhibition of adenosine deaminase activity by several benzodiazepines and nucleoside transport inhibitors

	Adenosine Deaminase Activity (nmol adenosine/min)	% Inhibition*
Control(no inhibitor)	8.38 ± 0.22	
Dimethyl Sulfoxide(1.25%)	8.12 ± 0.04	0
Flunitrazepam(100 μM)	7.67 ± 0.45	6
Hydroxynitrobenzylthioguanosine(12 μM)	7.33 ± 0.11	10
Diazepam(100 μM)	7.11 ± 0.19	12
Clonazepam(100 μM)	6.77 ± 0.34	17
Dipyridamole(100 μM)	6.21 ± 0.22	24

* Dimethyl sulfoxide(1.25%) was present in each of the test compound solutions. Therefore, the rate of adenosine deamination in the presence of dimethyl sulfoxide was used as the control (0% inhibition) value for the calculation of % inhibition of adenosine deaminase activity by the test compounds.

Results are the means ± s.e.mean from four experiments.

inhibition at concentrations of 100 μM (Table 29). Similarly, hydroxynitrobenzylthioguanosine (12 μM) did not significantly inhibit the activity of adenosine deaminase (Table 29).

IV

DISCUSSION

IV. DISCUSSION

High affinity, saturable binding sites for the nucleoside transport inhibitor NBMPR have been demonstrated on cells of various types (Lauzon & Paterson, 1977; Wohlhueter *et al.*, 1978; Paterson *et al.*, 1980; Dahlig-Harley *et al.*, 1981), including human erythrocytes (Pickard *et al.*, 1973; Cass *et al.*, 1974; Cass & Paterson, 1976; Jarvis *et al.*, 1982b). Occupation of these sites by NBMPR has been correlated with the inhibition of nucleoside transport (Cass *et al.*, 1974; Lauzon & Paterson, 1977), and other recognized nucleoside transport inhibitors such as dipyridamole have been reported to competitively inhibit the site-specific binding of NBMPR to HeLa cells, and erythrocytes (Cass & Paterson, 1976; Jarvis & Young, 1980; Paterson *et al.*, 1980).

The present study further explores the characteristics of the NBMPR binding site in erythrocytes, and describes the characteristics of a similar high affinity NBMPR binding site in mammalian CNS membranes. The demonstration that many nucleoside transport inhibitors inhibit the binding of NBMPR to both human erythrocytes, and CNS membranes further supports the suggestion that NBMPR binds to transport inhibitory sites on nucleoside transporters (Jarvis & Young, 1980). Furthermore, inhibition constants for several agents derived from the measurement of the inhibition of uridine influx and from competition experiments with NBMPR binding in erythrocytes, are similar. This indicates that inhibition of the binding of NBMPR by a test compound is predictive of the ability of that compound to inhibit nucleoside transport in human erythrocytes (Hammond *et al.*, 1983).

A. Nucleoside Transport in Fresh and Stored Human Erythrocytes

The equilibrium dissociation constant (K_D) of 0.31 nM determined in the present study for the binding of NBMPR to fresh human erythrocytes compares well with the affinity of NBMPR for its sites in other cell types (Lauzon & Paterson, 1977; Paterson *et al.*, 1980; Dahlig-Harley *et al.*, 1981), and with previously reported values for human erythrocytes (Pickard *et al.*, 1973; Cass *et al.*, 1974; Cass & Paterson, 1976; Jarvis *et al.*, 1982b). Erythrocytes obtained from stored blood (3 weeks at 4°C in citrate-phosphate-dextrose solution) had sites with an affinity for NBMPR which was 3-fold lower than those in fresh erythrocytes; the number of binding sites per cell, however, was similar in both fresh and stored cells.

Zero-*trans* uridine influx, and uridine equilibrium exchange influx studies in erythrocytes (Jarvis *et al.*, 1983) indicated that the V_{max} of uridine influx was 3-fold lower in stored erythrocytes relative to fresh erythrocytes, and these studies also implied that the mobility of the loaded and empty nucleoside carriers differed by about 6- and 12-fold in fresh and stored blood, respectively. Since both stored and fresh erythrocytes have similar numbers of NBMPR binding sites, the decrease in the V_{max} value for zero-*trans* influx in stored erythrocytes, relative to that in fresh cells, may be due to a decreased mobility of the unloaded nucleoside carrier rather than to a decrease in the number of functional nucleoside transporters (see Table 30). These results suggest that cellular nucleoside transport activity might be regulated, in some instances, by varying the mobility of the loaded and unloaded carriers. The lower mobility of the unloaded nucleoside carrier in stored erythrocytes may be associated with a

TABLE 30 Comparison of NBMPR binding constants and kinetic data for uridine zero-*trans* and equilibrium exchange influx in human erythrocytes from fresh and stored blood

		Fresh hRBC	Stored hRBC
NBMPR binding	B_{max} (molecules/cell)	11000 ± 600	12000 ± 1100
	K_D (nM)	0.31 ± 0.02	0.97 ± 0.13
Uridine* zero-*trans* influx	V_{max} (mmol/l H_2O per h)	116 ± 14	35 ± 4
	K_m (mM)	0.17 ± 0.02	0.13 ± 0.03
Uridine* equilibrium exchange influx	V_{max} (mmol/l H_2O per h)	462 ± 60	355 ± 41
	K_m (mM)	0.76 ± 0.07	1.10 ± 0.10

* Taken from Jarvis *et al.*,(1983).

Results are the means ± s.e.mean from at least 3 experiments.

conformation change in the transporter complex. This change may also affect the NBMPR binding site, leading to the observed decrease in affinity of this site in stored cells for NBMPR and other nucleoside transport inhibitors (as in Table 2).

B. Species Differences in Erythrocyte Nucleoside Transport

It has previously been determined that nucleoside transport capability in sheep, human, and pig erythrocytes depends upon the number of functional transporters in the cell membrane rather than on changes in transport capacity of individual carriers; each transporter has a constant translocation capacity of 140 to 180 molecules of uridine/site/s (Jarvis & Young, 1982).

The present results along with concurrently determined uridine influx kinetics (Jarvis *et al.*, 1982a), extend these studies to common laboratory animals including dog, rat, mouse, and rabbit erythrocytes (see Table 31). Both V_{max} values for uridine influx, and B_{max} values for the binding of NBMPR differed by as much as 40-fold among erythrocytes of the various species, although the apparent affinity of uridine (K_m), and NBMPR (K_D) for the nucleoside transporter were similar in each case (approximately 0.2 mM for uridine entry at 22°C, and 0.1 to 1 nM for NBMPR, respectively). Assuming that each NBMPR molecule binds to a single nucleoside transporter, translocation capacities calculated for the nucleoside transporter of human, rabbit, rat, and mouse erythrocytes were similar, and ranged from 123 to 190 molecules of uridine/site/s. These values compare favourably with the translocation capacities calculated previously for the nucleoside transport system of pig, sheep, and human erythrocytes (Jarvis & Young,

TABLE 31 Comparison of B_{max} values for the binding of NBMPR with V_{max} values for zero-*trans* uridine influx in erythrocytes from various species

Species	NBMPR B_{max} (molecules/cell)	Uridine V_{max} * (10^{19} X mol/ cell per s)	Translocation capacity (molecules/ sites per s)
Human	11000 ± 600	26 ± 3	142 ± 28
Rabbit	9500 ± 800	29 ± 2	190 ± 22
Pig	5100 ± 400*	11 ± 2	128
Mouse	3300 ± 500	6.8 ± 0.8	123 ± 48
Rat	300 ± 30	0.7 ± 0.1	140 ± 29
Guinea pig	27 ± 4 *	0.07 ± 0.01	155 ± 12
Dog	not detectable	not detectable	-

* Taken from Jarvis *et al.*, (1982a).

Results are the means ± s.e.mean from at least 3 experiments.

1982, and see Table 31). A similar relationship between the binding of NBMPR and uridine transport capability has been observed in guinea pig erythrocytes (Jarvis *et al.*, 1982a) although, due to a low transport capability, and therefore a low number of transport sites in these cells, NBMPR binding parameters had to be determined using erythrocyte membranes rather than intact cells (nonspecific binding of NBMPR is lower in haemoglobin-free erythrocyte membranes relative to intact cells). Dog erythrocytes do not possess high affinity binding sites for NBMPR, nor do they display saturable uridine or adenosine transport capability. This finding supports a previous conclusion that high affinity NBMPR binding to erythrocyte membranes is a specific association of NBMPR with functional nucleoside transport sites (Jarvis & Young, 1980).

The low transport and NBMPR binding capacity of dog and guinea pig erythrocytes is consistent with data obtained by Van Belle (1969a & b) who showed that, unlike many other species, guinea pig and dog erythrocytes were not involved in the metabolism of circulating adenosine. Similarly, adenosine uptake (combined passive diffusion and carrier-mediated transport) by dog erythrocytes was unaffected by the nucleoside transport inhibitor lidoflazine (Van Belle, 1970b), which suggests that adenosine accumulation by dog erythrocytes in that study was due only to passive diffusion.

This demonstration of substantial differences in nucleoside transport rates among erythrocytes from commonly used laboratory animals bears significantly on attempts to understand the influence of erythrocytes on nucleoside concentrations in the circulation. Thus, in biological systems where the erythrocyte transport system can influence blood concentrations of adenosine and other nucleosides, significant species differences should be expected in the metabolism of these agents, and in their ability to produce effects via interactions with extracellular receptors (for example, see Van Belle, 1969b). Similarly, the apparent potency of the adenosine receptor antagonist theophylline may also be influenced by the removal of adenosine from the vicinity of its receptors by the nucleoside transport mechanism (Clanachan & Muller, 1980).

The efficacy of nucleoside transport inhibitors as modulators of adenosine concentration in the extracellular space is also dependent upon the quantitative importance of the nucleoside transport system as a means of nucleoside removal. If, as has been suggested by Roos and Pfleger (1972), erythrocytes inactivate adenosine released into the coronary circulation, then the effects of the vasoactive inhibitors of nucleoside transport (such as dipyridamole or dilazep) should be expected to be significantly greater in humans than in dogs, guinea pigs, or rats. The rabbit would appear to possess an erythrocytic nucleoside transporter density that is more comparable to that of man than would the dog, guinea pig, or rat. In the dog, commonly used in coronary circulation studies, adenosine is not transported into erythrocytes, but may be inactivated by other cellular elements of blood. Van Belle (1969a & b) has proposed that platelets may perform this function in dog and guinea pig blood.

These findings demonstrate that the higher nucleoside transport capacity of rabbit, human, mouse, rat, and guinea pig erythrocytes relative to dog erythrocytes is related to the cell surface density of high affinity NBMPR binding sites (see Table 31). Species differences in the nucleoside transport capacity of erythrocytes are therefore due to differences in the cellular number of functional nucleoside transport sites, rather than to differences in the kinetic properties of the transport system.

Species differences in nucleoside uptake have also been observed in other cells. For instance, rat heart tissue accumulates adenosine by a transport-dependent mechanism at a much slower rate than does guinea pig heart tissue (Barker & Clanachan, 1982), and the rate of adenosine uptake by platelets is also species dependent (Van Belle, 1969a & b; Doni, 1981).

C. NBMPR Binding Sites in Guinea Pig CNS Membranes

Saturable, high affinity binding sites for the nucleoside transport inhibitor NBMPR also exist on membranes prepared from guinea pig brain. These sites appear to possess characteristics similar to the NBMPR binding sites on plasma membranes of human erythrocytes. Occupation of the latter sites by NBMPR has been correlated with inhibition of nucleoside transport (Cass *et al.*, 1974). Binding of NBMPR to cortical membrane sites was rapid, reversible, and dependent upon the pH and temperature of the incubation mixtures. The binding of NBMPR to guinea pig cortical membranes was linear with protein concentration up to 0.5 mg/ml protein, and no specific binding of NBMPR was observed in the absence of protein indicating that the binding was to the membrane protein, and not to assay tubes, or filters. The deviation from linearity observed when using greater than 0.5 mg/ml protein could be due to: 1) an endogenous inhibitor of the binding of NBMPR, possibly a purine, 2) aggregation of the synaptosomes, which is known to occur in media of high ionic strength (Gray & Whittaker, 1962), therefore physically occluding some of the binding sites, or 3) depletion of [^{3}H]NBMPR from the assay medium. The latter is the most likely case since 0.8 mg protein bound 160 fmol of [^{3}H]NBMPR in these experiments which reduced the initial [^{3}H]NBMPR concentration of 1.0 nM to a final free [^{3}H]NBMPR concentration of 0.84 nM. This was considered to be a significant degree of depletion.

Mass law analysis (Scatchard plot) of the site-specific binding data indicated that NBMPR bound to a single class of high affinity sites in guinea pig CNS membranes. The dissociation constant for NBMPR at these sites, calculated independently from saturation, competition, and kinetic experiments, ranged from 0.10 to 0.25 nM. These values are similar to those obtained for the site-specific binding of NBMPR in a variety of cell types, including erythrocytes from humans, and several other mammalian species. The apparent affinity of NBMPR for this site was dependent, to a minor degree, upon the termination procedure used in the binding assay. When centrifugation was used, NBMPR displayed a 0.25 nM affinity for the site, a 2.5-fold lower affinity than that observed when the assay was terminated by filtration; the total number of binding sites, however, was similar in each case (approx. 300 fmol/mg protein). Centrifugation for 4 min at 12,000 g in an Eppendorf microcentrifuge at room temperature, as performed in these experiments, produced a transient increase in the temperature of the assay mixture, and since NBMPR binding is temperature sensitive, this temperature increase may be sufficient to slightly decrease the affinity of the site for NBMPR. A filtration procedure, on the other hand, would not be expected to change the assay temperature.

Therefore, for binding systems which are extremely temperature sensitive, filtration, rather than centrifugation, may be the better means by which to separate the 'bound' from the 'free' radioligand.

Most of the NBMPR binding complexes in the CNS appear to be protein in nature as heating, trypsin, and alkaline pH eliminated 80% of the site-specific binding of NBMPR. Interestingly, neither the heat nor the trypsin inactivation procedures (which should have been of a sufficient duration to produce a maximum effect) eliminated all binding. These treatments did not affect 20% of the NBMPR binding sites which indicated that these sites were either not protein, or were in a relatively more stable conformation within the membrane. These results suggest heterogeneity of NBMPR binding sites in guinea pig CNS membranes, an hypothesis which will be discussed in greater detail in a subsequent section.

Subjecting the membrane preparation to a hypotonic medium reduced the site-specific binding of NBMPR by 36%, a proportion too large to be attributed solely to transport-mediated intracellular trapping of NBMPR. A 0.5 mg synaptosomal membrane protein pellet corresponds to approximately 20 μl aqueous volume, and assuming that 50% of this is intracellular space (10 μl, as a high estimate), the intracellular concentration of NBMPR would have to be 5.4 nM in order to account for the increased binding of NBMPR to intact membrane vesicles (synaptosomes). This is much higher than the concentrations of NBMPR used in these binding assays; also, NBMPR is not considered to interfere with phosphorylation reactions (Cass & Paterson, 1977; Paterson *et al.*, 1977b), and as the nucleoside transport system is nonconcentrative, it is unlikely that NBMPR is being 'trapped' within the synaptosomes.

Indeed, there is no reason to believe that NBMPR is even a substrate for the nucleoside transport system. An alternate possibility is that the lipid environment of the NBMPR binding complex in guinea pig CNS membranes is critical to binding activity and lysing the membranes causes local disruptions in the lipid bilayer structure.

Subcellular distribution: High affinity binding sites for NBMPR are uniformly distributed (per mg protein) throughout all subcellular membrane fractions with the exception of the mitochondrial fraction which had a low number of sites. This distribution is similar to that reported in rat brain subfractionation studies (Marangos *et al.*, 1982b). The lower density of binding sites (per mg protein) for NBMPR observed in the mitochondrial fraction may be attributable to synaptosomal contamination which is known to occur in this fraction with the preparation method used in these experiments (Marchbanks, 1975). NBMPR sites are therefore unlike many other CNS drug recognition sites as they are not necessarily localized close to synaptic junctions. This is not surprising as nucleosides are present in all types of cells and therefore a membrane-located nucleoside transport system would be expected to be ubiquitous.

Regional distribution: A marked regional heterogeneity in the distribution of NBMPR sites was found in guinea pig brain. As these sites appear to be similar to those in erythrocytes, where NBMPR binding sites represent functional nucleoside transporters, their non-uniform distribution in guinea pig brain suggests that differences may exist in the capacity of membranes in various brain areas to transport nucleosides. The highest density of sites was found in the brain stem regions, and the lowest density was located in the hippocampus. The distribution of the NBMPR binding sites appears unrelated to that for adenosine receptors. The latter, identified by the site-specific binding of 2-chloroadenosine, have a low density in spinal cord, hypothalamus, and pons/medulla, and a high density in hippocampus (Williams & Risley, 1980). The distribution of nucleoside transport sites, identified by the site-specific binding of NBMPR, also differs from that of the 'neuronal' benzodiazepine recognition sites (Mohler & Okada, 1978) which suggests that the 'neuronal' benzodiazepine recognition sites and the nucleoside transport sites are distinct entities. These results do not support the hypothesis of Phillis and coworkers (Phillis *et al.*, 1980; Wu *et al.*, 1981) that the 'neuronal' benzodiazepine binding sites are part of the nucleoside transport complex in the CNS and that benzodiazepines are producing their anxiolytic actions through an inhibition of the nucleoside transport system. Inhibitors of the binding of NBMPR, such as dipyridamole and diazepam, displayed no marked regional differences in their affinities for the NBMPR binding sites.

Assuming that NBMPR does bind specifically to the CNS nucleoside transport system (*vide infra*), this demonstration of regional differences in nucleoside transporter density may have important consequences in the measurement of drug action in the CNS. Significant regional differences should be expected in the apparent potency of adenosine receptor agonists and antagonists (Clanachan & Muller, 1980) as the concentration of agonist in the vicinity of its extracellular receptors may be influenced by its rate of removal by the transport system. Similarly, regional variations should be expected in the efficacy of nucleoside transport inhibitors as modulators of extracellular nucleoside concentrations. For instance, papaverine, a nucleoside transport inhibitor, has been shown to have its greatest effect on CNS blood flow in lower brain areas (pons and medulla) of the rat (Conway & Weiss, 1980). These results suggest that nucleoside transport inhibition has a greater effect on extracellular adenosine concentrations in the pons and medulla than in, for example, cerebellum. Therefore, a relatively higher density of nucleoside transporters may be present in the lower brain stem areas of rat brain. These results are consistent with the distribution of NBMPR binding sites in both rat brain (Marangos *et al.*, 1982b), and guinea pig brain.

D. Inhibitors of NBMPR Binding

1) General comments: Inhibitors of NBMPR binding display the same affinity for the NBMPR site in guinea pig brain as they do for the NBMPR site in human erythrocytes; in the latter system, NBMPR binds only to functional nucleoside transporters (Jarvis & Young, 1980), and inhibition of NBMPR binding reflects the potency of a compound as an inhibitor of nucleoside transport (Hammond *et al.*, 1983). Therefore, in the following section, inhibitors of the binding of NBMPR will often be discussed without reference to the particular binding system used (human erythrocytes or guinea pig CNS membranes). All compounds which inhibited the binding of NBMPR did so in an apparently competitive manner, and the K_i values determined directly by double reciprocal plot analysis were similar to the corresponding K_i values calculated from inhibitor IC_{50} values (see Table 32); slight differences between these two K_i estimates may be attributed to depletion of [^{3}H]NBMPR in

TABLE 32 Comparison of K_i values derived from double reciprocal plots with those calculated from IC_{50} values

	K_i double reciprocal plot analysis (μM)	K_i calculated from* IC_{50} value (μM)
Lorazepam	84 ± 9	87 ± 20
Chlordiazepoxide	59 ± 5	74 ± 20
Diazepam	9.8 ± 1.7	14 ± 2
Papaverine	4.2 ± 1.5	4.9 ± 0.3
	(nM)	(nM)
Lidoflazine	35 ± 4	35 ± 5
Dipyridamole	4.5 ± 0.2	5.2 ± 0.4
Hexobendine	1.8 ± 0.1	1.7 ± 0.3
Dilazep	0.9 ± 0.1	1.0 ± 0.1

All experiments were performed using stored human erythrocytes

* K_i values calculated according to the equation $K_i = \dfrac{IC_{50}}{1 + (L/K_D)}$

where L is the concentration of NBMPR(1.0 nM) used in these experiments to determine inhibitor IC_{50} values and K_D is the equilibrium dissociation constant for NBMPR in stored human erythrocytes.

Results are the means ± s.e.mean from at least 6 experiments.

the experiments determining inhibitor IC_{50} values.

2) Coronary vasodilators: Several coronary vasodilators are believed to produce relaxation of coronary vascular smooth muscle through an inhibition of nucleoside transport and consequently the potentiation of endogenous adenosine. These agents were potent inhibitors of the binding of NBMPR. Dipyridamole and lidoflazine inhibited the binding of NBMPR to HeLa cells in an apparently competitive manner with K_i values of 30 nM and 300 nM, respectively (Paterson *et al.*, 1980). These results are consistent with those values obtained in the present study with erythrocytes and CNS membranes. Competitive inhibition of the binding of NBMPR by these agents is surprising considering the structural dissimilarity between them and NBMPR (see Appendix 1).

The order of potency of these compounds as inhibitors of NBMPR binding (dilazep > hexobendine > dipyridamole > lidoflazine > papaverine) is similar to their order of potency for potentiation of adenosine-elicited accumulation of cyclic AMP (Huang & Daly, 1974), and their order of potency as adenosine uptake inhibitors (Kolassa *et al.*, 1978b; Mustafa, 1979)(see Table 33). However, contrary to the latter studies (Kolassa *et al.*, 1978b; Mustafa, 1979), in the present study, dilazep and hexobendine were slightly more potent than dipyridamole as inhibitors of the binding of NBMPR. This discrepancy may be due to the rapid degradation of dilazep and hexobendine by ester hydrolases (Schriewer & Rauen, 1972; Schaumloffel & Clausnitzer, 1972; Kolassa *et al.*, 1977; Kolassa *et al.*, 1978c) which may be present in the experimental assay systems used by Kolassa *et al.*, (1978b), and Mustafa (1979). This would result in an underestimation of the

TABLE 33 Comparison of the inhibition of the binding of NBMPR in fresh human erythrocytes with the inhibition of adenosine uptake in cardiac cells and the inhibition of uridine influx in fresh human erythrocytes by recognized nucleoside transport inhibitors.

	Inhibition of NBMPR binding to hRBC K_i (nM)	Inhibition of adenosine uptake in cardiac cells IC_{50} (nM)[*]	Inhibition of uridine influx in hRBC K_i (nM)[†]
Dilazep	0.29 ± 0.05	45	0.13
Hexobendine	1.9 ± 0.2	100	-
Dipyridamole	1.9 ± 0.4	40	1.0
Lidoflazine	42 ± 6	450	-
Papaverine	3500 ± 600	2000	-

[*] From Mustafa(1979)

[†] Jarvis, Hammond, Paterson & Clanachan, unpublished.

NBMPR binding results are the means ± s.e.mean from 6 experiments.

apparent potencies of dilazep and hexobendine. Significant metabolism of dilazep and hexobendine by ester hydrolases probably occurs in the present study, though possibly not to the same degree as in the assay systems described above, since inclusion of physostigmine, an ester hydrolase inhibitor, in the binding assay significantly increased the apparent affinities of dilazep and hexobendine for the NBMPR binding sites.

Verapamil, a coronary vasodilator which is believed to act independently of an inhibition of nucleoside transport, does not inhibit the binding of NBMPR.

The high degree of correlation between the inhibition of the binding of NBMPR by these coronary vasodilators, and their potency as adenosine uptake inhibitors supports previous suggestions that NBMPR binds to transport inhibitory sites of the nucleoside transport system (Jarvis & Young, 1980), and that inhibition of the binding of NBMPR reflects the ability of a compound to inhibit nucleoside transport (Hammond *et al.*, 1983). Recent data by Barker and Clanachan (1982) and Williams and Clanachan (1983) have shown that orders of potency for inhibition of the binding of NBMPR, inhibition of adenosine accumulation, and potentiation of adenosine action in guinea pig heart, are similar. The ability of these coronary vasodilators to compete with NBMPR for binding sites suggests that these agents are producing their inhibition of nucleoside transport in a manner which is similar to that of NBMPR.

The mechanism by which dipyridamole inhibits nuclesoide uptake has been a matter of dispute. Competitive (Scholtissek, 1968; Plagemann, 1971; Lips *et al.*, 1980; Jarvis *et al.*, 1982b), mixed

(Turnheim *et al.*, 1978), and non-competitive (Meunier & Morel, 1978; Rogler-Brown & Parks, 1980) dipyridamole inhibition kinetics have been reported. Dipyridamole competitively inhibits the binding of NBMPR and, therefore, may inhibit nucleoside transport in the same manner as NBMPR, but NBMPR itself has been described as a competitive, partially competitive, and non-competitive inhibitor of nucleoside uptake (Cass & Paterson, 1977; Eilam & Bibi, 1977; Eilam & Cabantchik, 1977; Paterson *et al.*, 1977a & b; Turnheim *et al.*, 1978; Wohlhueter *et al.*, 1978). A common assumption in the kinetic analysis of both inhibition of nucleoside transport and NBMPR binding by dipyridamole, and transport inhibition by NBMPR, has been that the concentration of free inhibitor in the medium is the same as the total (free + bound) inhibitor concentration of the cell suspension. However, recent experiments have demonstrated this not to be the case for NBMPR inhibition of nucleoside transport in erythrocytes (Jarvis *et al.*, 1982b). Furthermore, dipyridamole can inhibit the transport of anions, purine bases, and sugars in various cell systems (Plagemann & Richey, 1974), and these sites offer the potential for further depletion of dipyridamole from incubation media.

The influence of radioligand and inhibitor depletion on radioligand binding parameters and inhibition kinetics is exemplified in the present study using human erythrocytes. Failure to correct for NBMPR depletion resulted in an underestimation of the apparent affinity of NBMPR for the high affinity binding sites. This discrepancy was more pronounced when a higher number of ligand binding sites were present in the assay system (i.e., increasing cell number from 1.5 to 3 X 10^7 cells/ml doubled ligand depletion). Dipyridamole depletion could not be corrected for in this study, but the influence of this depletion on the kinetics of dipyridamole inhibition of the binding of NBMPR can be observed upon increasing the number of cells used in the assay. At 'low' cell concentrations (1.5 X 10^7 cells/assay), dipyridamole was an apparent competitive inhibitor of the binding of NBMPR, whereas, if twice the number of cells were used (resulting in twice as much dipyridamole depletion), dipyridamole displayed apparently non-competitive inhibition kinetics. All inhibition experiments performed in the current study used a low number of cells thereby minimizing inhibitor depletion. Hopefully, this allowed an accurate determination of the affinities of drugs for the NBMPR binding sites.

3) Nucleosides: Several nucleosides, which are substrates for the nucleoside transport system, also competitively inhibited the binding of NBMPR with the following order of potency: adenosine > inosine > thymidine > guanosine > uridine > cytidine. Although these agents appeared to be relatively weak inhibitors of the binding of NBMPR, their inhibition constants are similar to their Michaelis Menten constants (K_m) for transport in cultured cells and human erythrocytes (Berlin & Oliver, 1975; Plagemann & Richey, 1974; Paterson, 1979; Chello *et al.*, 1983)(see Table 34). Furthermore, as has been reported previously (Jarvis *et al.*, 1982), the K_i for inhibition of the binding of NBMPR by uridine is similar to the K_m value for uridine equilibrium exchange influx in erythrocytes.

It is not yet established whether inhibition of nucleoside transport by NBMPR derives from the latter binding at the permeation site, or at some other site on the transporter. The finding that several

TABLE 34 Comparison of inhibition constants(K_i) for inhibition of NBMPR binding and inhibition of adenosine transport by nucleosides

	Inhibition of NBMPR binding to CNS membranes K_i (μM)	Inhibition of* adenosine transport in L1210 cells K_i (μM)
Adenosine	123 ± 17	209 ± 37
Inosine	325 ± 32	566 ± 63
Thymidine	398 ± 68	605 ± 92
Guanosine	425 ± 32	958 ± 110
Uridine	940 ± 165	1106 ± 150
Cytidine	3000 ± 300	1449 ± 394

* From Chello *et al.*,(1983); data shown is for the 'low-affinity' nucleoside transport system in L1210 cells. A 'high-affinity' transport system was also reported which had substantially different affinities for various nucleosides

Results are the means ± s.e.mean of at least 4 determinations.

K_i values for the inhibition of nucleoside transport by nucleosides, which are substrates for the transporter, approximate their Michaelis-Menten constants (K_m) for the transport system.

nucleosides were competitive inhibitors of the binding of NBMPR is consistent with the notion that NBMPR may bind at or near the permeant site of the nucleoside transport system. However, it is also possible that inhibitor-induced conformation changes in the transporter may have resulted in 'competitive' inhibition plots (*vide infra*). 2-Chloroadenosine, a potent adenosine receptor agonist, also competitively inhibited the binding of NBMPR with a potency (K_i = 14 μM) which was nine times greater than that of adenosine (Table 17). This indicates that 2-chloroadenosine has affinity for the nucleoside transport system, but, as its pharmacological actions are not potentiated by nucleoside transport inhibitors (Muller & Paton, 1979), it may not be a substrate for the transporter, although this hypothesis has not been tested directly. 2-Chloroadenosine inhibits the transport of adenosine in L1210 cells with a K_i value of 24 μM (Sirotnak *et al.*, 1983). This provides further evidence that inhibition of the binding of NBMPR by a test compound is predictive of that compound's ability to inhibit nucleoside transport (Hammond *et al.*, 1983).

Recognition sites for NBMPR are distinct from extracellular adenosine receptors as the adenosine receptor antagonist, theophylline, did not inhibit the binding of NBMPR. Also, the affinity of 2-chloroadenosine at the NBMPR sites (14 μM) is significantly lower than that reported previously (K_D = 2.6 nM) for extracellular adenosine receptors (Williams & Risley, 1980). Furthermore, as discussed earlier, the CNS distribution of NBMPR binding sites differs from that of adenosine receptors. The purine nucleotides, ATP and GTP, were poor inhibitors of the binding of NBMPR and the small degree of inhibition observed may have been due to their conversion to the corresponding nucleosides.

4) Benzodiazepines: Inhibition of nucleoside transport, which might be expected to potentiate the extracellular effects of adenosine and other nucleosides, has been invoked in postulates about the central (Phillis *et al.*, 1980), and peripheral (Clanachan & Marshall, 1980b) actions of the benzodiazepines. The present study demonstrated that benzodiazepines do inhibit the binding of NBMPR to human erythrocytes and guinea pig CNS membranes. K_i values for benzodiazepine inhibition of the binding of NBMPR in erythrocytes were similar to those determined for benzodiazepine inhibition of zero-*trans* influx, zero-*trans* efflux, and equilibrium exchange influx of uridine in human erythrocytes (Hammond *et al.*, 1983)(see Table 35) indicating that inhibition of the binding of NBMPR by benzodiazepines is predictive of their abilities to inhibit nucleoside transport. The affinities of benzodiazepines for the NBMPR sites also correlate with their abilities to inhibit nucleoside uptake in guinea pig cardiac muscle (Barker & Clanachan, 1982) and rabbit vascular smooth muscle (Katsuragi & Su, 1982b), and to potentiate the effects of adenosine in cardiac (Clanachan & Marshall, 1980a; Kenakin, 1982) and smooth muscle (Clanachan & Marshall, 1980b).

Uridine transport studies (Hammond *et al.*, 1983) indicate that, although benzodiazepines are competitive inhibitors of equilibrium exchange influx of uridine, they are non-competitive inhibitors of zero-*trans* uridine influx and efflux. These results are consistent with the notion that benzodiazepines, like nucleoside permeants, are capable of binding to both the inward and outward facing conformations of the transport mechanism (Deves & Krupka, 1978). This is in contrast to NBMPR, which has been shown to bind preferentially to the

TABLE 35 Comparison of inhibition constants(K_i) for inhibition of NBMPR binding and inhibition of equilibrium exchange uridine influx in fresh human erythrocytes by benzodiazepines

	Inhibition of NBMPR binding to hRBC K_i (μM)	Inhibition of equilibrium* exchange uridine influx in hRBC K_i (μM)
Ro 5-4864	2.2 ± 0.6	8
Diazepam	6.8 ± 1.1	11
Clonazepam	24.1 ± 4.8	40
Lorazepam	45.1 ± 6.9	83

* From Hammond *et al.*,(1983).

Results are the means ± s.e.mean from at least 6 determinations.

outward facing conformation of the nucleoside transport system (Jarvis *et al.*, 1982b).

Benzodiazepines have a low affinity for the NBMPR site and are weak inhibitors of nucleoside transport relative to nucleoside transport inhibitors such as dipyridamole. Therefore, it is unlikely that significant inhibition of nucleoside transport would occur following the administration of anxiolytic doses of benzodiazepines. For example, an adult anxiolytic dose of diazepam (5 mg, p.o.) would result in a serum concentration of 0.1 to 1 μM (Hillestad *et al.*, 1974). In this range of concentrations, diazepam had no effect on the site-specific binding of NBMPR. Inhibition of nucleoside transport by some benzodiazepines at the higher plasma concentrations associated with the induction of anaesthsia (Rolly, 1976)(approximately 20 μM following diazepam 0.8 mg/kg, i.v.) may explain the well-documented coronary vasodilation observed following diazepam administration (Ikram *et al.*, 1973; Daniell, 1975). Diazepam also potentiates adenosine-induced coronary vasodilation *in vivo* (Clanachan & Marshall, 1980a). However, the ability to potentiate adenosine effects in tissues is not shared by all benzodiazepines. Diazepam potentiates adenosine effects on cardiac and smooth muscle *in vitro* (Clanachan & Marshall, 1980b), whereas lorazepam, which is approximately 5 times more potent than diazepam as an anxiolytic agent, was much weaker in its ability to potentiate adenosine effects in these experimental systems. The latter results correlate with NBMPR binding studies in which lorazepam was 6.5 times less potent than diazepam as an inhibitor of site-specific binding of NBMPR in human erythrocytes. Cardiovascular depression has long been recognized as a significant peripheral side effect of anaesthesia induction doses of diazepam, whereas this side effect is not associated with lorazepam administration (Rolly, 1976). This finding is understandable in light of the present results since lorazepam would be less effective than diazepam in potentiating the cardiovascular depressant effects of adenosine.

These findings may be particularly relevant for patients with ischaemic heart disease. Coronary vasodilators, such as dipyridamole, have been reported to produce 'coronary steal' in dogs (Wilcken *et al.*, 1971; Marshall & Parratt, 1973; Becker, 1978). 'Coronary steal' is defined as a diversion of blood flow away from a poorly perfused, but maximally dilated, area caused by general vasodilation of the non-ischaemic areas of the heart (Rowe, 1970). This situation is deleterious to the ischaemic regions of the heart. Diazepam, which is used as an anxiolytic and preanaesthetic medication, may produce coronary steal in patients with ischaemic heart disease via a potentiation of adenosine-induced vasodilation. Lorazepam, however, which is 5 times more potent an anxiolytic as diazepam, appears to be about 6 times less potent than diazepam in inhibiting nucleoside transport in erythrocytes and, therefore, lorazepam has a lower potential for producing significant coronary vasodilation. This means that lorazepam, at any given anxiolytic dose, would have less potential to produce coronary steal in patients with ischaemic heart disease.

Several studies have indicated that benzodiazepines inhibit the accumulation of adenosine in brain tissue (Mah & Daly, 1976; Traversa & Newman, 1979; Phillis *et al.*, 1981; York & Davies, 1982) and potentiate adenosine-mediated effects in the CNS (Phillis, 1979). Inhibition of adenosine uptake in rat cortical synaptosomes by benzodiazepines was shown to correlate with their clinical effectiveness as anxiolytics, and with their order of affinity for the 'neuronal' benzodiazepine recognition sites (Phillis *et al.*, 1981); these authors proposed that the 'neuronal' benzodiazepine receptors may actually be part of the CNS nucleoside transport system, and that nucleoside transport inhibition may play a role in the clinical effects of the benzodiazepines (Wu *et al.*, 1981). The order of affinity of the benzodiazepines for the nucleoside transport system of erythrocytes and CNS membranes (Ro 5-4864 > diazepam > clonazepam > oxazepam > lorazepam > flurazepam), as determined by inhibition of, (a) uridine transport in erythrocytes (Hammond *et al.*, 1983), and (b) binding of NBMPR in erythrocytes and CNS membranes, is significantly different from the order of affinity of these compounds for both the 'neuronal' and 'non-neuronal' benzodiazepine recognition sites (see Table 36). Therefore, the 'neuronal' benzodiazepine binding sites, which are assumed to be the receptors at which the benzodiazepines act to produce their CNS effects, are unrelated to the NBMPR sites associated with the nucleoside transport system. The benzodiazepines possess high affinity for the former (K_i for diazepam is 7.4 nM)(Mohler & Okada, 1978), but low affinity for the latter (K_i for diazepam is approx. 10 μM). Furthermore, the proposed endogenous ligand for the 'neuronal' benzodiazepine recognition sites, βCCE (Braestrup *et al.*, 1980), and the potent benzodiazepine receptor antagonist, Ro 15-1788 (Hunkler *et al.*, 1981), had low affinities for the NBMPR sites, suggesting that these compounds are very weak inhibitors of nucleoside transport; these results find support in a recent study by Morgan *et al.*, (1983), who showed that Ro 15-1788 did not prevent benzodiazepine inhibition of adenosine

TABLE 36 Comparison of inhibition constants(K_i) for inhibition of NBMPR binding to guinea pig CNS membranes, inhibition of flunitrazepam binding to human CNS membranes, and inhibition of diazepam binding to guinea pig heart tissue by dipyridamole and several benzodiazepines

	Inhibition of NBMPR binding to guinea pig CNS membranes K_i (μM)	Inhibition of[*] flunitrazepam binding to human CNS membranes K_i (μM)	Inhibition of[†] diazepam binding to guinea pig heart tissue K_i (μM)
Dipyridamole	0.011 ± 0.002	(0.230)[¶]	0.024
Ro 5-4864	5.4 ± 0.6	100	0.0022
Diazepam	16 ± 2	0.027	0.048
Clonazepam	34 ± 5	0.0013	2.3
Lorazepam	52 ± 14	0.0010	-

[*] From Speth *et al.*,(1978) - 'neuronal' benzodiazepine recognition site.

[†] From Davies and Huston(1981) - 'non-neuronal' benzodiazepine site.

[¶] From Davies *et al.*,(1980) using rat CNS membranes.

Results for the inhibition of NBMPR binding are means ± s.e.mean, n = 6.

uptake into rat cerebral cortex synaptosomes. A further distinction between the NBMPR binding sites and the 'neuronal' benzodiazepine binding sites comes from the observation that the NBMPR site on erythrocytes and CNS membranes displayed a selectivity for the (-) isomer of the benzodiazepine stereoisomeric pair Ro 11-6896(+) and Ro 11-6893(-), whereas the 'neuronal' benzodiazepine site prefers the (+) isomer (Mohler & Okada, 1978). In addition, the 'neuronal' benzodiazepine binding sites and the NBMPR sites exhibit different regional distributions in the CNS.

NBMPR sites are also unrelated to the 'non-neuronal' benzodiazepine recognition sites. Ro 5-4864, which is 1000-fold more potent than clonazepam at the 'non-neuronal' benzodiazepine site, is only 6- to 10-fold more potent than clonazepam at the NBMPR binding site. As well, Ro 5-4864 displays a 1000-fold lower affinity for the NBMPR site than for the 'non-neuronal' benzodiazepine site (see Table 36).

The NBMPR binding sites may be related to the 'micromolar' benzodiazepine recognition sites reported to exist in rat brain (Bowling & DeLorenzo, 1982). Benzodiazepines display comparable affinities (µM) for each site, and the order of affinity fo these agents for the NBMPR site and for the 'micromolar' benzodiazepine site are similar (see Table 37); only Ro 5-4864 displays significantly different potencies for each site, and this may be due to methodological differences. Binding of benzodiazepines to the 'micromolar' benzodiazepine sites has been correlated with their ability to inhibit maximum electric shock-induced convulsions and from this it was suggested that these sites may be involved in the modulation of neuronal excitability and anticonvulsant activity. These are functions which have also been

TABLE 37 Comparison of the inhibition of the binding of NBMPR in stored human erythrocytes with the inhibition of diazepam binding to the "micromolar" benzodiazepine recognition site in rat CNS membranes, by several benzodiazepines

	Inhibition of NBMPR binding to hRBC K_i (μM)	Inhibition of[†] diazepam binding to "micromolar" sites K_i (μM)
Ro 5-4864	2.2 ± 0.6[*]	491
Flunitrazepam	13 ± 1	42
Diazepam	13 ± 2	85
Clonazepam	51 ± 5	182
Oxazepam	58 ± 3	243
Chlordiazepoxide	75 ± 20	393
Flurazepam	>300	1120

[*] Obtained using fresh human erythrocytes.

[†] From Bowling and DeLorenzo(1982).

K_i values for the inhibition of the binding of NBMPR were calculated from the corresponding IC_{50} values and represent mean ± s.e.mean of 3 experiments performed in duplicate.

attributed to adenosine in the CNS. Therefore, these authors may have been looking at diazepam binding to the NBMPR recognition sites in rat CNS membranes (see Table 37) which may be located on transport inhibitory elements of the nucleoside transport system.

Since the binding of NBMPR has been correlated, in some systems, with inhibition of nucleoside transport (Cass *et al.*, 1974; Lauzon & Paterson, 1977), the present demonstration that several benzodiazepines competitively inhibit NBMPR binding, indicates that benzodiazepines may, indeed, inhibit nucleoside transport. However, the apparent low affinity of the benzodiazepines for transport inhibitory sites, and the lack of correlation between their affinities for the nucleoside transport inhibitory sites and their clinical potency as anxiolytics, indicates that inhibition of nucleoside transport is unlikely to be involved in the central anxiolytic mechanism of action of the benzodiazepines.

The apparent discrepancy between the results of the present study and those of Phillis and coworkers (Phillis *et al.*, 1981; Wu *et al.*, 1981) may be due to their difficulty in measuring initial rates of the uptake of adenosine in rat cortical synaptosomes. As long incubation times were used (30 s), apparent uptake rates may have been influenced by intracellular enzymatic reactions (adenosine kinase and/or adenosine deaminase), or by nucleoside entry by passive diffusion followed by subsequent metabolic trapping. Because rates of nucleoside transport, in some instances, exceed rates at which internalized nucleoside molecules are enzymatically transformed, time-courses for cellular uptake of nucleosides are complex. This has necessitated use of rapid sampling techniques to obtain the definitive time-courses of cellular nucleoside uptake which are needed to obtain initial rates of nucleoside uptake. The latter measure transport. Thus, interpretation of cellular nucleoside uptake rates as transport rates (and of inhibition of nucleoside uptake as transport inhibition) requires that the uptake rates be demonstrably initial rates. Therefore, the results of Phillis and coworkers could be explained by an inhibition of intracellular adenosine metabolism by the benzodiazepines. Since benzodiazepines do not appear to affect adenosine deaminase activity, the effect of low concentrations of the benzodiazepines (< 1 μM) on adenosine uptake may be mediated through an inhibition of adenosine kinase.

5) Phenothiazines: The phenothiazines are another class of drugs which have been proposed to produce some of their central effects through an inhibition of the cellular accumulation of adenosine (Phillis & Wu, 1981b). However, these agents were also weak inhibitors of the site-specific binding of NBMPR, indicating that they may have low affinity for the nucleoside transport system.

The specificity of the inhibition of the binding of NBMPR by these high concentrations of benzodiazepines and phenothiazines was confirmed by the observation that a variety of putative neurotransmitters, neurotransmitter antagonists, and CNS-active agents were without significant effects on the binding of NBMPR.

E. Thermodynamic Analysis of NBMPR Binding and Inhibitor Interactions

The interaction of compounds with their specific binding sites produces characteristic perturbations in the thermodynamic parameters of the assay systems. A study of the magnitude and direction of these changes may provide insights into the conformational changes associated with ligand-binding site interactions. In the current study, the thermodynamic constants associated with drug interactions with the NBMPR binding site complex were measured. These experiments provided another point of comparison between the erythrocytic NBMPR site and the NBMPR binding site in CNS membranes.

The binding of NBMPR is driven by a large loss in enthalpy. The free energy of binding (ΔG) is dominated by ΔH and the entropic term ($T\Delta S$), which is subject to considerable estimation error, makes a small contribution to ΔG. The thermodynamic characteristics of the binding of dipyridamole and diazepam are quite different from those of NBMPR. Although ΔG is similar, the binding reaction appears to be driven by an increase in entropy which overcomes the gain in enthalpy.

Similar thermodynamic differences have been observed for agonist and antagonist binding at β-adrenoceptors (Weiland *et al.*, 1980), and similar arguments may be used to interpret the binding interactions at the NBMPR binding site of the nucleoside transporter. The thermodynamic parameters for NBMPR binding, which reflect only net changes of the complex system, indicate that an increase in entropy, resulting from the hydrophobic association step, may be followed by a large decrease in enthalpy due to NBMPR-induced conformational changes in the transporter system. The loss in enthalpy effectively reverses the gain in entropy. The binding of dipyridamole or diazepam, on the other hand, may not induce such conformational changes in the drug-transporter complex.

The temperature dependence of adenosine binding to transport inhibitory sites was similar to that of NBMPR in that its interaction with the transport system resulted in a large loss in enthalpy and, therefore, like NBMPR, adenosine may induce a conformational change in the transport complex. This was not unexpected since adenosine is a substrate for the nucleoside transport system. However, unlike NBMPR, adenosine interaction with the transport complex also caused a large decrease in entropy. These results suggest that 'loading' the carrier with a substrate increases the 'order' within the nucleoside transport system. This conclusion is supported by uridine influx and efflux studies which indicated that the 'loaded' carrier is 6 times more mobile, and therefore more functionally organized, than the 'unloaded' carrier in fresh human erythrocytes (Jarvis *et al.*, 1983).

F. Kinetic Analysis of Dissociation of the NBMPR-Binding Site Complex

Another means by which to characterize a binding site is to study the rates at which agents dissociate from the site under various assay conditions.

Semi-log plots of the dissociation of NBMPR from its sites in human erythrocytes were monophasic, which indicated that NBMPR bound to an apparent single class of sites in these cells. In guinea pig CNS membranes, on the other hand, where the NBMPR dissociation was biphasic, NBMPR may have been dissociating from two different types (or conformations) of binding sites. NBMPR site heterogeneity in the CNS will be further discussed in a subsequent section. The rate of dissociation of NBMPR from its high affinity sites was dependent upon the displacing agent used. Uridine and adenosine, which are both substrates for the nucleoside transporter, produced the fastest dissociation rates, and dipyridamole, a compound structurally dissimilar to nucleoside permeants, produced the slowest rate of NBMPR dissociation. Large concentrations of non-radiolabelled NBMPR produced an intermediate rate of [^{3}H]NBMPR dissociation. These results are consistent with a model in which the NBMPR-transporter complex exists in two interconvertible conformations which differ with respect to their rates of dissociation. In this model, dipyridamole stabilizes the complex in the slow dissociation state, whereas uridine and adenosine stimulate the conversion to a fast dissociation state. The fact that adenosine and uridine are substrates for the transporter suggest that the dissociation of NBMPR from the transporter complex occurs at a higher rate when the transport site is occupied by a permeant molecule; the 'loaded' nucleoside carrier is more mobile than the 'unloaded' carrier (Jarvis *et al.*, 1983). If one considers the NBMPR-transporter complex to be an equilibrium between two conformations, then agents which interact with the transport system, but do not significantly affect this equilibrium, may produce a dissociation rate of NBMPR which is intermediate between that produced by dipyridamole (which changes the equilibrium in favour of the slow dissociation state) and that produced by nucleoside substrates (which change the equilibrium in favour of the fast dissociation state). This may be the explanation for the intermediate [^{3}H]NBMPR dissociation rate induced by non-radiolabelled NBMPR and cogeners. Since the dissociation rate constant (k_2) of NBMPR varies with the displacer used, the equilibrium dissociation constant (K_D) of NBMPR in guinea pig cortical membranes calculated from kinetic experiments as k_2/k_1 also varies with the compound used to obtain k_2. Nitrobenzylthioinosine 5'-monophosphate (cleaved to NBMPR by ecto 5'-nucleotidase) induces an initial dissociation of NBMPR of 0.167 min^{-1} in guinea pig cortical membranes; using this value, a K_D of 0.30 nM may be calculated. This value is similar to the K_D determined from mass law analysis of equilibrium binding data (0.25 nM). As the determination of K_D by mass law analysis does not require the presence of high concentrations of a compound which has the potential to induce a conformation change in the NBMPR binding site complex, this K_D would reflect the dissociation rate of NBMPR resulting from the spontaneous rate of conversion between the 'fast' and 'slow' dissociation states of the NBMPR-binding site complex at equilibrium. Therefore, the rate at which non-radiolabelled NBMPR induces the dissociation of [^{3}H]NBMPR appears to be dependent upon the spontaneous rate of conversion between the 'fast' and 'slow' dissociating conformations, NBMPR itself does not affect this equilibrium. This model, of course, is only one of many which could be devised given the available information.

It is not yet known whether NBMPR binds to the permeant site of the transport complex or a distinct, transport regulatory site (see Introduction). However, the model outlined above does not necessitate that NBMPR binds to the permeant site, but only requires that changes in transporter activity affect the NBMPR binding site complex which is a component of the nucleoside transport system.

The fast NBMPR dissociation rate induced by diazepam is not consistent with this model. Diazepam induced a NBMPR dissociation rate similar to that caused by adenosine, yet diazepam interacts with the NBMPR binding site complex in a manner which is thermodynamically similar to that of transport inhibitors such as dipyridamole and opposite to that of nucleoside transporter substrates such as adenosine (*vide supra*). Therefore, the possibility that the affinity of the NBMPR binding site is being regulated by one or more allosteric sites, which are independent from the permeant site, must be considered. An allosteric site would have affinity for dipyridamole, diazepam, and nucleosides. These agents may even be producing their apparent 'competitive' inhibition of the binding of NBMPR through interactions with this allosteric site. Alternatively, the dissociation rate of NBMPR may depend on membrane fluidity and, therefore, the ability of an agent to induce the dissociation of NBMPR from its specific high affinity sites may depend on a nonspecific effect on membrane lipids.

Regardless of which model, if any, is correct, NBMPR appeared to always dissociate from the same type of site conformation irrespective of the displacing agent used since the activation energy ($E_a(-)$) for NBMPR dissociation, determined from the temperature dependence of the dissociation rate (Arrhenius Plot), is independent of the actual displacing agent.

Evidence has been obtained for ligand-induced receptor conformation changes in many other systems (Burgen, 1981) including β-receptors (Weiland *et al.*, 1980), and benzodiazepine receptors (Quast & Mahlmann, 1982; Quast *et al.*, 1982). It has even been suggested that these conformational changes may provide a mechanism for modulation of receptor affinity (Burgen, 1981). A situation analogous to that seen in the present study with NBMPR has been described for the diazepam binding site in rat cortical membranes where the rate of dissociation of [^{3}H]diazepam from its high affinity binding sites was dependent upon the benzodiazepine used as the "displacer" (Chiu & Rosenberg, 1982). These investigators also invoked a ligand-induced conformation change as a possible interpretation of their data.

G. Does NBMPR Bind to the Nucleoside Transporter in Guinea Pig Brain?

The binding of NBMPR to specific, high affinity sites in erythrocyte membranes has been directly correlated with the inhibition of nucleoside transport in these cells, suggesting that NBMPR interacts specifically with components of the erythrocyte nucleoside transport system (Cass *et al.*, 1974). In many cell types, NBMPR seems to bind only to functional nucleoside transporters. Nucleoside-impermeable dog and sheep erythrocytes (Jarvis & Young, 1980; Jarvis *et al.*, 1982a), and nucleoside transport deficient mouse lymphoma cells (Cass *et al.*, 1981) do not possess high affinity NBMPR binding sites. Various nucleoside transport inhibitors inhibit the binding of NBMPR to human erythrocytes. The ability of these compounds to inhibit binding has been directly correlated with their ability to inhibit uridine influx and to potentiate the effects of adenosine in many different systems.

NBMPR binding sites in guinea pig cortical membranes display the same affinity for nucleoside transport inhibitors as does the NBMPR binding site in human erythrocytes, and the order of potency of these compounds for each site is similar to their order of potency as adenosine potentiators. Agents which are permeants of the nucleoside transport system, inhibit the binding of NBMPR to CNS membranes with K_i values which are similar to their K_m values for transport in various cell types. These results suggest that the binding site for NBMPR in guinea pig CNS membranes has the same characteristics as the site in human erythrocytes which has been shown to be part of the nucleoside transport complex. Indeed, a similar type of analysis has been used to distinguish β_1 and β_2 adrenoceptors, and H_1 and H_2 histamine receptors, that is, receptors were identified according to the order of affinity of agonists and antagonists for the sites (Lands *et al.*, 1967; Black *et al.*, 1972). Furthermore, the interaction of NBMPR with its binding sites in guinea pig brain displays the same thermodynamic properties as the interaction of NBMPR with the erythrocyte nucleoside transport complex. Similarly, the NBMPR dissociation kinetics are the same in both guinea pig cortical membranes and human erythrocytes. The rate of dissociation of NBMPR from its high affinity sites is dependent upon the displacing agent used and this relationship between dissociation rate and 'displacer' is identical in both erythrocytes and guinea pig CNS membranes. Therefore, the characteristics of the NBMPR binding sites in guinea pig brain are essentially identical to the characteristics of the NBMPR binding sites on the nucleoside transport complexes in erythrocytes. The numerous pieces of evidence presented here strongly suggest that NBMPR is binding to CNS nucleoside transport complexes.

Definitive evidence for NBMPR binding to the CNS nucleoside transporter can only be obtained through nucleoside transport studies similar to those previously performed using human erythrocytes, where the binding of NBMPR has been correlated with NBMPR-mediated inhibition of nucleoside transport, and inhibitors of the binding of NBMPR have been shown to have similar potencies as inhibitors of nucleoside transport (Cass *et al.*, 1974; Hammond *et al.*, 1983). In order to distinguish nucleoside transport from intracellular metabolism of nucleosides, initial rates of nucleoside flux must be measured which often requires time-courses of a few seconds. This makes transport studies rather complex in that a rapid 'stopping' method is needed which does not disrupt the intact cell. Transport studies in erythrocytes and cultured cells are often terminated by the addition of a potent transport inhibitor (such as NBMPR) and sedimentation of the cells through an oil layer in order to separate the intracellular from the extracellular radiolabelled nucleoside (Paterson *et al.*, 1981). CNS synaptosomes, unfortunately, are much more fragile than erythrocytes and cultured cells, and tend to lyse when sedimented through oil. Filtration may also be used to 'stop' the transport experiments, but this method does not prevent rapid efflux of the nucleoside during the washing of the filter. The filter must be washed in these experiments since the radiolabelled nucleoside associated with the unwashed filter can often greatly exceed that associated with the cellular material, which makes analysis of this data impossible. Furthermore, vacuum filtration has been observed to cause lysing of erythrocytes and may therefore also lead to rupture of the synaptosomal membrane. Since nucleoside transport is a non-concentrative process, the intracellular volume will dictate the maximum intracellular content of nucleoside accumulated during a transport experiment. Synaptosomes have very small intracellular volumes, therefore, unless large assay volumes are used, minute changes in the amount of nucleoside accumulated within the synaptosomes would be difficult to detect with any accuracy by traditional detection methods (i.e., liquid scintillation spectrometry).

The present studies on the binding of NBMPR in the CNS must be correlated with CNS nucleoside transport studies before it could be said with absolute conviction that NBMPR binds to, and consequently inhibits, the CNS nucleoside transport system. However, this information cannot be obtained until significant methodology problems are overcome for the measurement of initial rates of nucleoside flux in synaptosomes.

H. Species Differences in the Binding of NBMPR to CNS Membranes: Evidence for Binding Site Heterogeneity

There appear to be two forms of high affinity NBMPR binding sites in mammalian cortical membranes which differ with respect to their affinity for NBMPR and dipyridamole. Both forms of NBMPR sites were apparent in rabbit cortical membranes where mass law analysis of the site-specific NBMPR binding data resulted in non-linear Scatchard plots; one site had an affinity (K_D) for NBMPR of 4.1 nM, which was similar to the affinity of the NBMPR site in dog cortical membranes, and the other NBMPR site in rabbit cortex displayed a K_D of 0.32 nM, which was similar to that observed for NBMPR binding to guinea pig cortical membranes. These sites in rabbit cortex can be further distinguished with the use of dipyridamole. Dipyridamole exhibited biphasic concentration-inhibition plots for the inhibition of the binding of NBMPR in rabbit cortical membranes. One NBMPR site displayed an affinity for dipyridamole which was approximately 10,000 times higher ($IC_{50} \simeq 4$ nM) than the affinity of the other NBMPR site for dipyridamole ($IC_{50} \simeq 40$ μM). Diazepam, however, displayed monophasic concentration-inhibition plots and therefore, unlike dipyridamole, cannot be used to distinguish between the two types of sites.

Biphasic plots for the inhibition of NBMPR binding by dipyridamole were also obtained when using dog and guinea pig cortical membranes. These findings are not consistent with the apparently linear Scatchard plots (indicating a single class of sites) obtained for the binding of NBMPR to these membranes. Problems with the interpretation of Scatchard plots may account for these discrepancies. For instance, linear Scatchard plots, normally interpreted as a single class of sites, may also be obtained if the radioligand binds to two distinct sites with similar affinities. This, however, does not appear to be a problem in the present study as the affinity of the two NBMPR sites in rabbit cortex differed by more than 40-fold. A second possible reason for misinterpretation of Scatchard plots would arise if one of the two binding sites constituted a small proportion (i.e., less than 20%) of the total number of sites. Under these circumstances, a curvilinear Scatchard plot would not be readily apparent. This latter possibility may be the explanation for the discrepancies seen in the present studies. Data obtained from competition experiments measuring dipyridamole-mediated inhibition of the binding of NBMPR to dog and rabbit cortical membranes at different NBMPR concentrations indicated that the 'high' affinity dipyridamole site corresponds to the 'high' affinity NBMPR site in these membranes. When half of the NBMPR binding sites (≈ 300 fmol/mg) in dog cortical membranes (Figure 24, Table 24) were labelled with an 'average' K_D concentration of NBMPR (4.0 nM), dipyridamole displayed an IC_{50} of 7.5 nM at 32% of these sites (Figure 26, Table 27); therefore, 32% represents the proportion of 'high' affinity NBMPR binding sites labelled in these experiments. Since 'high' affinity NBMPR sites would be saturated by 4.0 nM NBMPR, it may be calculated that these sites only make up 16% of the total number of NBMPR binding sites in dog cortical membranes; a proportion which can be easily 'masked' by experimental error in a Scatchard analysis (see Figure 24).

The dissociation of NBMPR from its sites in guinea pig and rabbit cortical membranes occurs in a biphasic manner providing further evidence for the existence of two populations of NBMPR binding sites in CNS membranes. These latter results also indicate that the 'slow' dissociating NBMPR binding site complexes generally constitute less than 20% of the total number of NBMPR site complexes in rabbit cortical membranes, a value which is comparable to the proportion of 'high' affinity NBMPR sites in rabbit cortex determined by mass law analysis of NBMPR equilibrium binding data. Therefore, the difference in affinity of NBMPR for the two sites may be due, in part, to differences in the rate of dissociation of NBMPR from each binding site complex.

Hill coefficients of less than 0.75 obtained for diazepam inhibition of the binding of NBMPR to dog and rabbit cortical membranes (Table 25) are also indicative of multiple NBMPR binding sites.

Treatment of guinea pig cortical synaptosomes with trypsin, or incubation of these membranes for one hour at 60°C, eliminated 80% of the site-specific binding of NBMPR. The remaining 20% may represent the 'high' affinity type of NBMPR binding site discussed above which is either not protein in nature, or is in a relatively more stable conformation within the membrane.

Binding site heterogeneity implies either two or more distinct types of sites or two or more conformations of the same site.

Definitive evidence for the existence of two conformations of a single site, rather than distinct sites, can be obtained from experiments which measure the relative numbers of each type of binding site in one particular assay system under various conditions. If the relative proportions of these sites change with a particular membrane treatment, without a change being observed in the total number of sites or in the distribution of sites, then the possibility of two interconvertible binding site conformations may be indicated. Although such experiments were not undertaken in the present study, other evidence suggestive of multiple conformations of the NBMPR binding site was obtained. The rate of NBMPR dissociation from its sites in cortical membranes was biphasic and was also dependent upon the displacing agent used, which suggests that each 'displacer' differed in its ability to induce a conformation change in the NBMPR binding site complex to a state from which NBMPR is able to dissociate at a faster rate (*vide supra*). Furthermore, the proportion of the 'slow' dissociating to 'fast' dissociating NBMPR binding site complexes in rabbit cortical membranes was dependent upon the agent used to displace site-bound NBMPR. Even though each 'displacer' induced a different initial rate of NBMPR dissociation, the dissociation rate always decreased to a similar, but much slower, rate after 2 to 3 min of exposure to the high concentration of 'displacer', regardless of the quantity of site-bound NBMPR remaining at that time. These latter results provide strong evidence for the existence of two interconvertible NBMPR site conformations in rabbit cortical membranes.

The association rates (k_1) calculated for the binding of NBMPR to each class of sites in rabbit cortical membranes were similar, which further indicates that these sites are the same. The results obtained with rabbit cortical membranes suggest that the interaction of a ligand with a class of NBMPR sites causes a subsequent conformation change in the sites resulting in a change in dissociation rate and, therefore, a change in the apparent K_D of the interacting ligand for these sites.

It is not yet known whether NBMPR binds to the nucleoside transport system in CNS membranes. However, the NBMPR binding sites in guinea pig cortical membranes, which display high affinity for dipyridamole, have characteristics which are identical to those of the NBMPR binding sites in human erythrocytes where NBMPR binds only to a functional nucleoside transport system (*vide supra*). These results therefore suggest that the NBMPR binding sites in cortical membranes, which have high affinity for dipyridamole, are components of a nucleoside transport system similar to that in erythrocytes. In human erythrocytes, inhibition of the binding of NBMPR by a test compound is predictive of the ability of that compound to inhibit nucleoside transport (Hammond *et al.*, 1983).

Rat and mouse cortical membranes appear to contain only one class of high affinity NBMPR binding site and these sites display a low affinity for dipyridamole (Figure 25, Table 27). If this NBMPR binding site with low affinity for dipyridamole is simply a different conformation of the same site which can also display high affinity for dipyridamole, then a nucleoside transport system may exist in some mammalian cortical membranes which is less susceptible to inhibition by dipyridamole.

Other investigators have described a similar binding site for

NBMPR with low affinity for dipyridamole in rat cortical membranes (Marangos *et al.*, 1982b; Wu & Phillis, 1982a). The results of Marangos *et al.*, (1982b) indicated that dipyridamole had a K_i value of 283 nM for the inhibition of the binding of NBMPR to rat cortical membranes, a value which is similar to that determined in the present study for dipyridamole inhibition of the binding of NBMPR to rat cortical membranes (K_i = 404 nM). These values are much higher than the K_i value for dipyridamole-induced inhibition of the binding of NBMPR to guinea pig cortical membranes (K_i = 11 nM) and human erythrocytes (K_i = 1.9 nM). In another study, by Patel *et al.*, (1982), the nucleoside transport inhibitor hexobendine was also found to be a weak inhibitor of the binding of NBMPR to rat cortical membranes (K_i = 880 nM), 130 times weaker than as an inhibitor of the binding of NBMPR to guinea pig cortical membranes (K_i = 6.7 nM). Wu and Phillis (1982a) also described dipyridamole and hexobendine as being weak inhibitors ($<$ 50% inhibition at 10 μM) of the binding of NBMPR (10 nM) to rat cortical membranes.

The low potency of dipyridamole as an inhibitor of the binding of NBMPR in rat does not appear to be restricted to brain tissue as the high affinity binding sites for NBMPR in rat heart have also been shown to have a low affinity for dipyridamole (K_i = 276 nM)(Williams & Clanachan, personal communication). Therefore, dipyridamole and hexobendine may be poor inhibitors of nucleoside transport in the rat. This hypothesis finds considerable support in the literature. For example, in contrast to results obtained using guinea pig tissues, dipyridamole and hexobendine did not potentiate the negative chronotropic effects of adenosine in rat heart (Stafford, 1966; Kolassa *et* *al.*, 1971; Sakai *et al.*, 1981). Similarly, dipyridamole did not enhance adenosine-induced renal vasoconstriction in rats but significantly potentiated this effect of adenosine in pig, dog, and rabbit kidney (Sakai *et al.*, 1981). Furthermore, adenosine uptake by rat lungs and erythrocytes was only inhibited by dipyridamole and hexobendine at concentrations which were 100-fold higher than those required to inhibit adenosine uptake by guinea pig lungs and erythrocytes (Kolassa *et al.*, 1971; Kolassa & Pfleger, 1975). Dipyridamole and hexobendine were very weak inhibitors of adenosine uptake by rat heart, but were potent inhibitors of adenosine uptake by guinea pig heart (Hopkins & Goldie, 1971; Kolassa *et al.*, 1971; Barker & Clanachan, 1982). These agents were also poor inhibitors of adenosine uptake by rat brain synaptosomes (Wu *et al.*, 1981). These results suggest that the nucleoside transport system in rat tissues is much less susceptible to inhibition by dipyridamole and hexobendine than is the nucleoside transporter in other species such as guinea pig. Therefore, the present finding that dipyridamole was a weaker inhibitor of the binding of NBMPR in rat brain than it was in guinea pig brain is further evidence that NBMPR binds to the nucleoside transport system in the CNS, and that inhibition of the binding of NBMPR by a test compound is predictive of the ability of that compound to inhibit nucleoside transport.

It is of interest to note that a nucleoside transport system which is insensitive to inhibition by NBMPR has recently been described in some cell types (Lauzon & Paterson, 1977; Wohlhueter *et al.*, 1979; Paterson *et al.*, 1983; Belt, 1983). One of these cell types, Walker 256 cells (Paterson *et al.*, 1983), has normal transport capability, but possesses few, if any, high affinity binding sites for NBMPR. Therefore, at least three types of nucleoside transporters may coexist in mammalian cells: 1) a type which is sensitive to inhibition by low concentrations (< 10 nM) of NBMPR and dipyridamole, 2) another which has transport inhibitory sites with high affinity for NBMPR but low affinity for dipyridamole, and 3) a type of transporter which is not inhibitable by low concentrations of NBMPR.

The results of the present study and those in the literature provide conclusive evidence that NBMPR binds specifically to functional nucleoside transporters in erythrocytes. A type of NBMPR binding site exists in mammalian CNS membranes which displays characteristics identical to those of the erythrocyte NBMPR site. NBMPR, therefore, probably also binds to nucleoside transport inhibitory sites in the CNS. However, in the present experiments, multiple forms of NBMPR binding sites have been characterized in these membranes. This indicates that different types of nucleoside transporters may coexist in mammalian CNS membranes.

I. Summary

The nucleoside transport system of erythrocytes and CNS membranes has been studied with the high affinity probe NBMPR.

1) NBMPR bound with high affinity to a single class of sites in human, rabbit, mouse, rat, and guinea pig erythrocytes. The maximal binding capacity was species dependent, and was proportional to the maximal velocity of zero-*trans* uridine influx. Assuming that each NBMPR molecule bound to a single nucleoside transporter, translocation capacities, calculated for the transporter in each species, were similar (approximately 150 molecules of uridine/site per s, at 22°C). Therefore, species differences in the nucleoside transport capacity of erythrocytes among the species tested were due to differences in the cellular number of functional transporters, rather than to differences in the kinetic properties of the transport system.

In dog erythrocytes, which do not transport uridine or adenosine, high affinity NBMPR binding sites were not demonstrable. This supports the hypothesis that NBMPR binds only to functional nucleoside transporters in erythrocytes.

2) Constants for the inhibition of the binding of NBMPR by several agents, derived from competition experiments with NBMPR, were similar to those obtained from the direct measurement of the inhibition of uridine transport in human erythrocytes. Therefore, inhibition of the binding of NBMPR in this system by a test compound is predictive of the ability of that compound to inhibit nucleoside transport.

3) NBMPR also binds to high affinity sites in guinea pig CNS membranes. Regional differences in site density were apparent, but subcellular distribution of the sites was relatively uniform.

The binding of NBMPR to these CNS sites was dependent upon the pH and temperature of the incubation media.

4) NBMPR binding sites in guinea pig CNS membranes, and those in human erythrocytes, were compared.

a) Affinities for various drugs from several classes of nucleoside transport inhibitors (6-thiopurine nucleoside derivatives, coronary vasodilators, benzodiazepines, phenothiazines, β-carbolines) were similar in both systems.

b) Rates of association of the site-specific binding of NBMPR were rapid and dependent upon temperature in both systems. Dissociation of NBMPR from its sites, measured in the presence of non-radiolabelled NBMPR, was temperature dependent (activation energy of dissociation was approximately 98 kJ/mole). Also, in both systems, rates of dissociation of NBMPR, relative to those obtained using non-radiolabelled NBMPR, were slowed by dipyridamole and accelerated by adenosine, uridine, or diazepam.

c) The temperature dependence of the affinities of NBMPR, dipyridamole, adenosine, and diazepam for the NBMPR binding site in both human erythrocytes and guinea pig CNS membranes was determined. These studies revealed that the binding of NBMPR or adenosine was driven by a large loss in enthalpy, whereas the binding of dipyridamole or diazepam was driven by an increase in entropy which overcame a gain in enthalpy.

These latter studies (b and c) indicated that drugs differed in their abilities to induce conformational changes in the NBMPR site complex. Dipyridamole, for instance, tended to 'stabilize' the NBMPR binding site complex. Nucleoside transporter substrates, on the other hand, seemed to induce a conformational change in the site complex, possibly due to their ability to stimulate the nucleoside carrier.

The many similarities which were found between the characteristics of CNS NBMPR binding sites and those of the erythrocyte NBMPR sites, suggest that NBMPR binds to the nucleoside transport system in guinea pig CNS membranes in the same manner as in erythrocytes.

5) NBMPR binding sites appear to be distinct from adenosine receptors and 'neuronal' and 'non-neuronal' benzodiazepine sites.

Adenosine receptors and NBMPR sites displayed different regional distributions in the CNS, and the potent adenosine receptor agonist, 2-chloroadenosine, had a much lower affinity for the NBMPR site.

The order of affinity of benzodiazepines for the 'neuronal' benzodiazepine recognition site, which may be involved in the anxiolytic actions of benzodiazepines, is totally different from the order of affinity of these agents for the NBMPR site. Furthermore, most of the benzodiazepines tested displayed about 1000-fold lower affinity for the NBMPR site than for the 'neuronal' benzodiazepine site.

The NBMPR binding site is unlikely to be the 'non-neuronal' type of benzodiazepine recognition site due to, a) the low affinities of the benzodiazepines, and b) the relative lack of discrimination between Ro 5-4864 and clonazepam, for the NBMPR binding site.

6) The NBMPR binding sites may be related to the "micromolar" benzodiazepine recognition sites which have recently been characterized.

7) Several coronary vasodilator agents (dilazep, hexobendine, dipyridamole), which have been proposed to act by potentiating adenosine-mediated vasodilation, displayed high affinity ($K_i < 10$ nM) for transport inhibitory NBMPR sites in human and rabbit erythrocytes, and in guinea pig CNS membranes.

8) Several benzodiazepines, as mentioned above, also inhibited the binding of NBMPR. However, they were much less potent in that respect than nucleoside transport inhibitors such as dipyridamole.

This, and the fact that the order of potency of the benzodiazepines for the NBMPR site does not correlate with their order of potency as anxiolytic agents, suggests that nucleoside transport inhibition may not play a major role in the central anxiolytic mechanism of action of benzodiazepines.

However, higher concentrations of some benzodiazepines, such as those associated with the induction of anaesthesia, have the potential to produce effects, such as coronary vasodilation, via nucleoside transport inhibition.

This may explain the well documented coronary vasodilation that has been observed upon the administration of large doses of diazepam. This indicates that some, but not all, benzodiazepines have the potential to produce 'coronary steal' when used in patients with ischaemic heart disease.

9) Nucleosides, which are substrates for the nucleoside transport system, inhibited the binding of NBMPR in an apparently competitive manner, with K_i values similar to their K_m values for the transport system. These results indicated that NBMPR may bind at or near the permeant site of the nucleoside transport system.

10) The density of NBMPR sites within guinea pig brain displayed marked regional heterogeneity. Also, the membrane density of these sites varied among erythrocytes and CNS membranes of several commonly used laboratory animals (rat, mouse, guinea pig, rabbit, dog). Therefore, differences in the nucleoside transport capacity of these membranes may exist. This would be expected to influence the rate of nucleoside inactivation in the circulation and brain, leading to variations in the apparent potency of adenosine receptor agonists and antagonists. Similarly, differences should be expected in the efficacy of transport inhibitors as modulators of extracellular, and intracellular, nucleoside concentrations.

11) Multiple forms of NBMPR binding sites were demonstrated in mammalian CNS membranes. Each had high affinity for NBMPR ($K_D < 5$ nM). However, one class of NBMPR site displayed an affinity for dipyridamole which was 10000 - fold higher than that of another class of sites.

The relative proportion of these two classes of NBMPR sites in CNS membranes varied among species. Rabbit and dog CNS membranes contained both forms, whereas rat CNS membranes only contained a single class of sites which had low affinity for dipyridamole. These results may explain the relatively low potency of dipyridamole as an adenosine 'uptake' inhibitor, and as a potentiator of adenosine effects, in rat tissues.

Evidence obtained from analysis of drug-induced dissociation of NBMPR from its sites in several species, indicated that two interconvertible forms of a single class of NBMPR sites may exist, rather than two distinct types of binding sites. Therefore, both types of NBMPR binding sites may be associated with the nucleoside transport system of CNS membranes.

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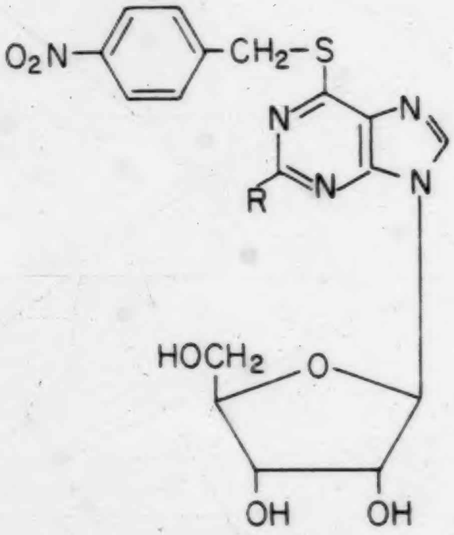
APPENDIX 1

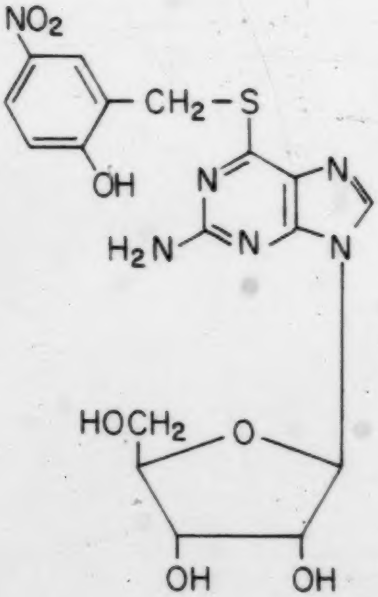
Chemical Structures

a) 6-Thiopurine nucleoside derivatives



Nitrobenzylthioinosine R = H

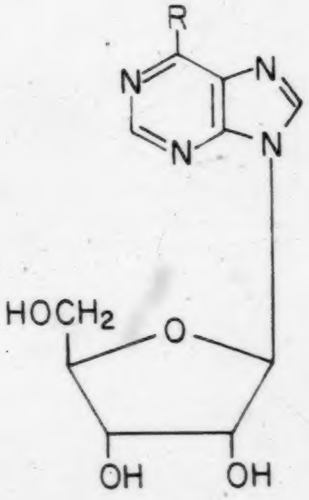
Nitrobenzylthioguanosine R = NH_2



Hydroxynitrobenzylthioguanosine

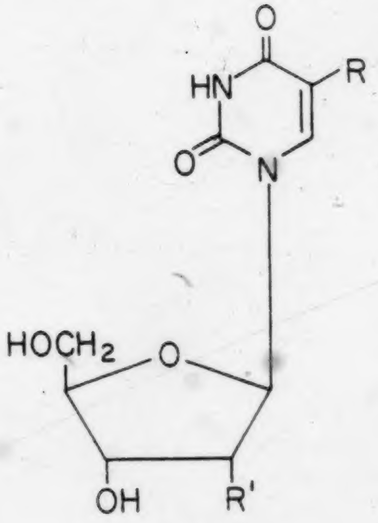
?

b) Nucleosides



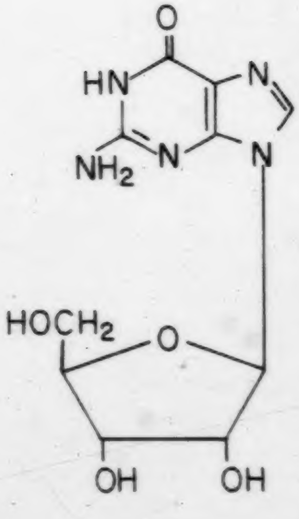
Adenosine R = NH_2

Inosine R = OH

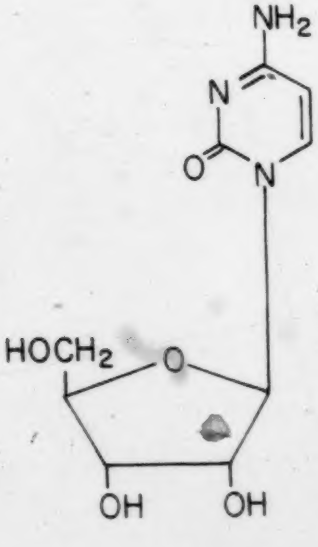


Thymidine R = CH_3
R'= H

Uridine R = H
R'= OH

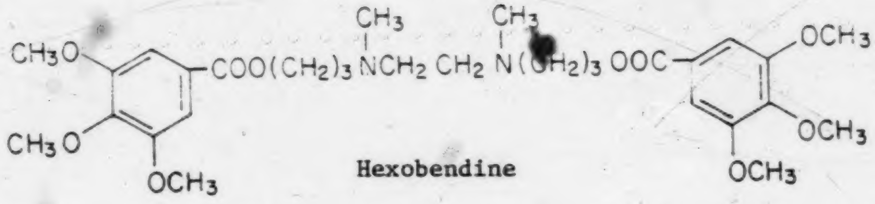


Guanosine

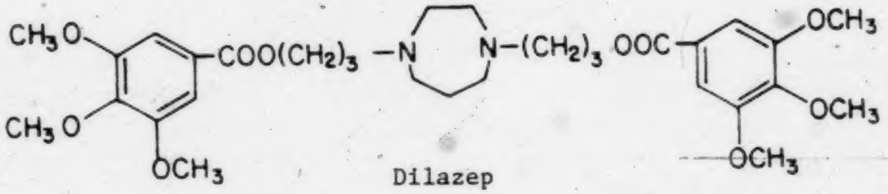


Cytidine

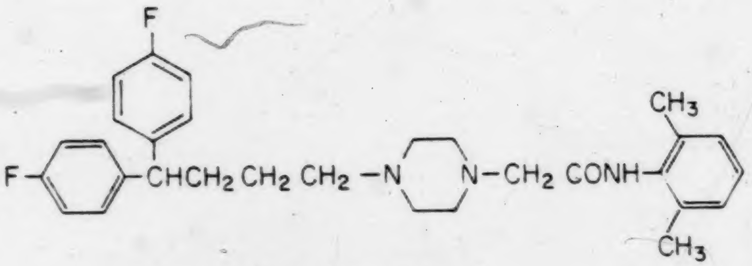
c) Coronary vasodilator agents



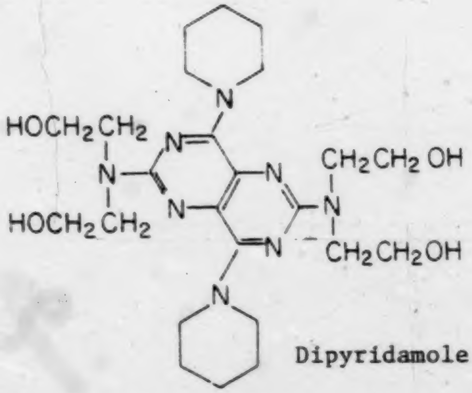
Hexobendine



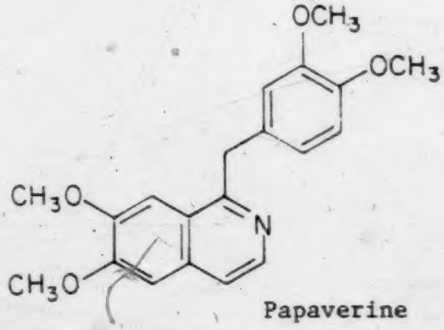
Dilazep



Lidoflazine

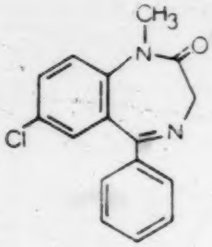


Dipyridamole

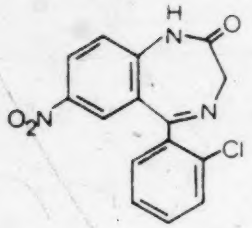


Papaverine

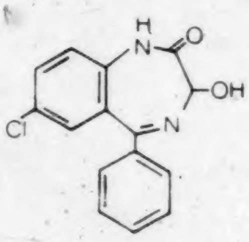
d) Benzodiazepines



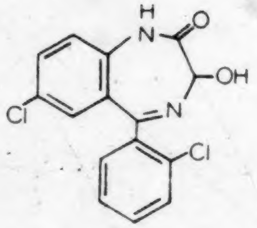
Diazepam



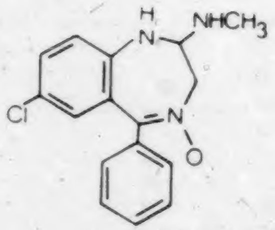
Clonazepam



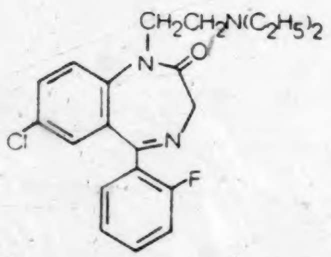
Oxazepam



Lorazepam



Chlordiazepoxide



Flurazepam

APPENDIX 2

Composition of Buffers and Liquid Scintillation Fluor

a) Composition of Dulbecco's Phosphate Buffered Saline(PBS)

NaCl	(137 mM)	8 g/l
$Na_2HPO_4 \cdot 7H_2O$	(6.3 mM)	1.7 g/l
KCl	(2.7 mM)	200 mg/l
KH_2PO_4	(1.5 mM)	200 mg/l
$MgCl_2 \cdot 6H_2O$	(0.5 mM)	100 mg/l
$CaCl_2 \cdot 2H_2O$	(0.9 mM)	135 mg/l

Adjusted to pH 7.4 with KOH (from Dulbecco & Vogt, 1954)

b) Composition of Krebs-Tris buffer

Nacl	(118 mM)	6.9 g/l
*TRIS	(15 mM)	1.8 g/l
KCl	(4.8 mM)	358 mg/l
$MgCl_2 \cdot 6H_2O$	(1.2 mM)	243 mg/l
$CaCl_2 \cdot 2H_2O$	(1.2 mM)	176 mg/l

*TRIS(Hydroxymethyl)Aminomethane

Adjusted to pH 7.4 with HCl (from Mohler & Okada, 1977)

c) Composition of liquid scintillation fluor--Tritisol(4 litres)

Benzoyl peroxide	13.3 g
*PPO	12 g
**POPOP	800 mg
Xylene	2300 ml
Triton X-100	1000 ml
Ethanol(98%)	560 ml
Ethylene glycol	140 ml

*PPO = 2,5-Diphenyloxazole

**POPOP = 1,4-Bis(5-phenyloxazol-2-yl)-benzene

APPENDIX 3

Liquid Scintillation Spectrometry

a) Sample preparation:

i) Centrifugation assays: Cell, or membrane, pellets were digested in 250 µl of 0.5 N KOH for a minimum of 5 h. The digested material was then transfered to a 6 ml scintillation vial(Minivial^{R}) with a pasteur pipette and neutralized with 100 µl of 1.25 N HCl. Tritisol (5 ml), prepared as described in Appendix 2, was added to the scintillation vial and, after 5 h(to decrease chemiluminescence and allow time for benzoyl peroxide in the Tritisol to bleach the erythrocyte samples), the samples were assayed for ^{3}H-radioactivity by liquid scintillation spectrometry in a Beckman LS230 liquid scintillation counter.

ii) Filtration assays: Filters were placed in 6 ml scintillation vials and dried at 55°C for greater than 12 h. They were then dissolved, by vigorous shaking, in 5 ml of Tritisol. These samples were allowed to sit for 12 h in order to minimize the amount of chemiluminescence and were then assayed for ^{3}H-radioactivitity by liquid scintillation spectrometry using a Beckman LS230 liquid scintillation counter.

b) Liquid scintillation counting parameters:

All samples were counted for a time sufficient to achieve a 1.5% counting error or for a maximum of 10 min. The degree of quenching in each sample was estimated by the sample channels ratio technique (B/A ratio). Data were obtained both on paper and punched tape via a teletype connection.

c) Preparation of quench correction curve:

A new quench correction curve was obtained every six months using tritiated water of known specific activity(corrected for isotope decay) as the standard radiation source. A known amount of tritiated water (≈ 20 µl) was added to each liquid scintillation vial and acetone, used as a quenching agent, was then added to the vials(10 to 250 µl/vial) followed by 5.0 ml of Tritisol. Samples were allowed to stand for 5 h and were then assayed in a Beckman LS230 liquid scintillation counter, using the counting parameters outlined above. Sample counting efficiency was then plotted against the respective B/A ratio to obtain a linear function as shown in Figure 29. The equation of this line was incorporated into a computer program (See Section F.1 and Appendix 6) to provide a means for rapid quench correction of data.

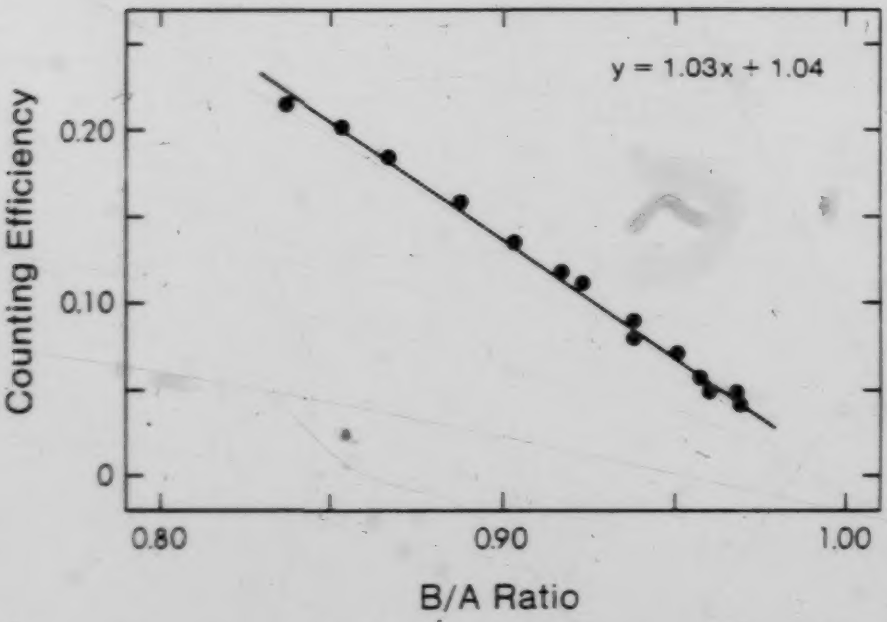


FIGURE 29 Representative quench correction curve.

APPENDIX 4

Determination of Protein Concentrations

This protein determination was based on the method of Lowry *et al.*(1951).

a) Reagents: A. 2% Na_2CO_3 in 0.1 N NaOH

B. 0.5% $CuSO_4 \cdot 5H_2O$

C. 1% NaK Tartrate

D. Folin-Ciocalteu reagent(2 N) diluted 1:2.5 with distilled water

E. A 50:1:1 solution of A:B:C made up as required

b) Method: The samples were prepared in a volume of 1.0 ml Krebs-Tris buffer(Appendix 2). Assays were initiated by the addition of 2.0 ml of reagent E, immediately mixed, and incubated at room temperature for 17.5 min. At the end of this time, 0.2 ml of reagent D was added, mixed immediately and incubated at room temperature for 50 min. The optical density of the sample was then determined at 650 mu in a Gilford spectrophotometer with subtraction of a 'blank' (no protein) value.

c) Preparation of standard curve: Bovine serum albumin(BSA) was used as a standard. A stock solution of 2 mg BSA/ml was prepared in Krebs-Tris buffer and a 1:10 dilution of this stock was made at time of the assay. A 9 point standard curve(40 to 200 μg/ml BSA in a final volume of 1 ml) was constructed each time a membrane protein sample was to be determined(See Figure 30 for example standard curve). The BSA equivalent concentration of the sample solution could then be

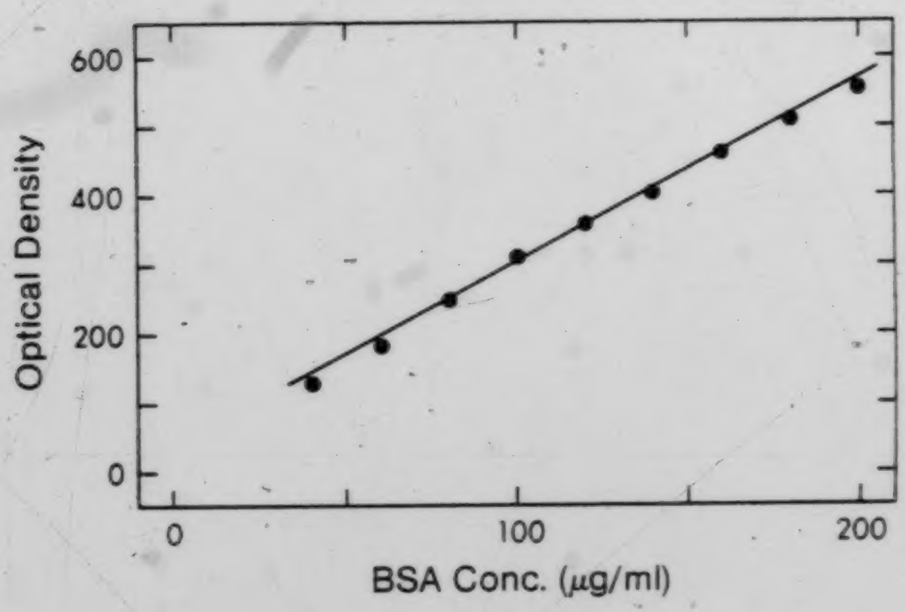


FIGURE 30 Representative standard(bovine serum albumin) curve for the determination of protein concentrations.

determined from the standard curve, and since the crystalline BSA was 6% water(i.e. 94% protein), the protein concentration equals 0.94 times the BSA equivalent concentration.

APPENDIX 5

Spectral Data

Compound (in H_2O)	λ_{max} (nm)	$\varepsilon \times 10^{-4}$ (Molar absorbance)
Nucleosides[*]		
Adenosine	260	1.51
Cytidine	271	0.91
Guanosine	252.5	1.37
Inosine	248.5	1.22
Thymidine	267	0.97
Uridine	261	1.01
Purine Nucleoside Derivatives		
Nitrobenzylthioinosine[†]	290	2.51
Nitrobenzylthioguanosine[¶]	283	2.88
Hydroxynitrobenzylthioguanosine[†]	314	1.90
Nucleotides		
ATP[*]	259	1.54
GTP[§]	252	1.37

[*] From the Merck Index(1976)

[†] From Lynch *et al.* (1981)

[¶] From Noell and Robins(1962)

[§] From Volkin and Cohn(1954)

All spectral data shown were obtained at a pH of between 7 and 8.

APPENDIX 6

Computer Programs

a) Data analysis program--JIM1

```
C *** For Jim Hammond - May 1st, 1980 ***
C *** Calculate DPMs from CPMs using quench correction ***
      INTEGER*2 LENHF
      LOGICAL*1 LINE(80)
      REAL CPM, DPM, X, Y
      INTEGER CNUMB, DNUMB, ICPM1, ICPM2, IX1, IX2
      CALL FTNCMD(´ASSIGN 0=TAPE.JIM1´,18)
      N = 1
      WRITE(6,100)
C *** Read next line in 0=TAPE.JIM1 & scan for ´(´ in A=999999.9( ***
1     CALL READ(LINE,LENHF,0,LNUM,0,&999)
      CALL FINDST(LINE,80,´(´,1,1,I,&5)
      I = I - 8
C *** ´(´ shouldn´t be close to the end of the line ***
      IF (I .GE. LENHF) GOTO 5
      CNUMB = 6
C *** Convert A=999999.9 to binary (999999 first, then .9) ***
      CALL DTB(LINE(I),ICPM1,CNUMB,DNUMB,´=´,&5)
      I = I + 7
      CNUMB = 1
      CALL DTB(LINE(I),ICPM2,CNUMB,DNUMB,´.´,&5)
C *** Add 999999 and .9 to get CPM ***
      CPM = ICPM1 + 0.1*ICPM2
C *** Back up from end of line to convert S=0.999 similarly ***
      I = LENHF - 4
      CNUMB = 1
      CALL DTB(LINE(I),IX1,CNUMB,DNUMB,´=´,&6)
      I = I + 2
      CNUMB = 3
      CALL DTB(LINE(I),IX2,CNUMB,DNUMB,´.´,&6)
C *** Add the 0. and the .999 together to get X (the ESR) ***
      X = IX1 + 0.001*IX2
      Y = -1.030*X + 1.040
C *** Calculate DPM <-- CPM / Y and print DPM and its no. ***
4     DPM = CPM / Y
      WRITE(6,101) N, DPM
      GOTO 7
5     WRITE(6,102) N
      GOTO 7
6     WRITE(6,103) N
7     N = N + 1
      GOTO 1
999   WRITE(6,104)
```

```
      CALL CMD('LIST TAPE.JIM1',14)
      STOP
100   FORMAT(//' DPM'/)
101   FORMAT(' ',I3,4X,F10.1)
102   FORMAT(' ',I3,4X,' (Cannot find CPM)')
103   FORMAT(' ',I3,4X,' (Cannot find R)')
104   FORMAT(/' *** DONE ***'//)
      END
```

b) Data analysis program--JIM2

```
C *** For Jim Hammond - August 1, 1980 ***
C *** Calculate DPMs using quench correction (subr. CLCDPM),
C *** Do other calculations & save % control values for APL t-tests.
C
      LOGICAL*1 NAME(64), LETTER
      LOGICAL EQUC
      LOGICAL*1 PCLN(12) /´P´,´C´,´9´,´9´,´9´,´=´,´R´,´E´,
     * ´A´,´D´,´ ´,´ ´/
      INTEGER*2 LENHF /12/
      INTEGER EXPNO, CNUMB /3/, DNUMB
      REAL CELLNO, DPM(20), SA, FACTOR, BKG, TOTBND, NSBND, SPBND
      REAL MLCELL, PCNTRL(200), AVGPC, TBCPM(3), CPM, TBX(3), X
      COMMON /C1/ CPM, X

      CALL FTNCMD(´ASSIGN 0=TAPE.JIM2´,18)
      CALL FREAD(-2,´PREFIX´,´ ´)
      CALL FREAD(-2,´ENDLINE´,´STREAM´)
C
C *** Get input and calculate background, SA, factor, bound values
C
      WRITE(6,100)
      READ(5,102,END=99) (NAME(J), J=1,64)
      WRITE(6,104)
      CALL FREAD(5, ´I:´, EXPNO)
      WRITE(6,106)
      CALL FREAD(5, ´R:´, GELLNO)
      DO 10 J=1,20
10    CALL CLCDPM(DPM(J),0,&999,&9999)
      SA = 1.0 / ( (DPM(1)+DPM(2)+DPM(3)) / 3.0 )
      FACTOR = (6.025E11/CELLNO) * SA
      BKG = (DPM(4)+DPM(5)+DPM(6)+DPM(17)+DPM(18)+DPM(19)+DPM(20)) / 7.0
      TOTBND = ( ( (DPM(11)+DPM(12)+DPM(13)) / 3.0 ) - BKG ) * FACTOR
      WRITE(6,108) TOTBND
      READ(5,110) LETTER
      IF (EQUC(LETTER,´N´) .OR. EQUC(LETTER,´n´)) GOTO 30
C
C *** Get new CPM and X values from user (to calculate TOTBND)
C
      WRITE(6,112)
      CALL FREAD(5, ´2R V:´, TBCPM,3, TBX,3)
      DO 20 J=1, 3
      CPM = TBCPM(J)
      X = TBX(J)
      K = J + 10
20    CALL CLCDPM(DPM(K),1,&999,&9999)
      TOTBND = ( ( (DPM(11)+DPM(12)+DPM(13)) / 3.0 ) - BKG ) * FACTOR
30    NSBND = ( ( (DPM(14)+DPM(15)+DPM(16)) / 3.0 ) - BKG ) * FACTOR
      SPBND = TOTBND - NSBND
C
C *** Output global stuff
C
      WRITE(6,200) EXPNO, (NAME(J), J=1,64)
      WRITE(6,202) CELLNO, SA
      WRITE(6,204) FACTOR, BKG
      WRITE(6,206) TOTBND, NSBND
      WRITE(6,208) SPBND
      DO 35 J=1,19
35    WRITE(6,210) J, DPM(J)
      J = 20
      WRITE(6,212) J, DPM(J)
C
C *** Calculate molecules/cell, percent control & avg. % control 'til
EOF
C
      I = 21
40    CALL CLCDPM(DPM(1),0,&999,&9999)
      MLCELL = ( (DPM(1) - BKG) * FACTOR ) - NSBND
      PCNTRL(I) = (MLCELL/SPBND) * 100.0
      AVGPC = PCNTRL(I)
      WRITE(6,214) I, DPM(1), MLCELL, PCNTRL(I)
      I = I + 1
      CALL CLCDPM(DPM(1),0,&999,&9999)
      MLCELL = ( (DPM(1) - BKG) * FACTOR ) - NSBND
      PCNTRL(I) = (MLCELL/SPBND) * 100.0
      AVGPC = (AVGPC + PCNTRL(I)) / 2.0
      WRITE(6,216) I, DPM(1), MLCELL, PCNTRL(I), AVGPC
      I = I + 1
      GOTO 40

999   WRITE(6,500)
      CALL CMD('LIST TAPE.JIM2',14)
C
C *** Write expt. number & % control values for input to APL
C
      CALL FTNCMD('ASSIGN 7=PC.JIM2',16)
      CALL EMPTYF(7)
      WRITE(7,300)
      I = I - 1
      WRITE(7,302) (PCNTRL(J), J=21,I)
      CALL FTNCMD('ASSIGN 8=APL.JIM2',17)
      CALL BTD(EXPNO,PCLN(3),CNUMB,DNUMB,' ')
      IF (EQUC(PCLN(4),' ')) CALL MOVEC(4,' PC',PCLN)
      IF (EQUC(PCLN(3),' ')) CALL MOVEC(3,' PC',PCLN)
      CALL WRITE(PCLN,LENHF,2,3000,8)
      CALL CMD('SOU APL.JIM2',12)
99    STOP
9999  WRITE(6,900)
      STOP

100   FORMAT(//'Enter experiment name ("|" shows maximum length)',
     * ' |')
102   FORMAT(64A1)
104   FORMAT('&Enter experiment number: ')
106   FORMAT('&Enter cell number: ')
```

```
108   FORMAT(/´Total bound = ´,F8.0,´ molecules/cell.´/
     * ´&Do you wish to substitute? ´)
110   FORMAT(A1)
112   FORMAT(/´Enter three CPM´´s then three X´´s, spaces between:´)
200   FORMAT(´1Expt. No.´,I3,´ - ´,64A1)
202   FORMAT(´0 Cell No. = ´,G9.3/´0 S.A. = ´,G9.3)
204   FORMAT(´0 Factor = ´,F4.2/´0 BKG = ´,F4.0)
206   FORMAT(´0 Total Bound = ´,F6.0/´0 N.S. Bound = ´,F6.0)
208   FORMAT(´0 Specific Bound = ´,F6.0/´- DPM´/)
210   FORMAT(´ ´,I3,F12.0)
212   FORMAT(´ ´,I3,F12.0,´ Molecules/Cell % Control ´,
     * ´Avg. % Control´)
214   FORMAT(´ ´,I3,F12.0,8X,F8.0,8X,F5.0)
216   FORMAT(´ ´,I3,F12.0,8X,F8.0,8X,F5.0,8X,F5.0)
300   FORMAT(´ 0 0 0 0 0´)
302   FORMAT(10F5.0)
500   FORMAT(//´ *** End of paper tape file ***´//)
900   FORMAT(//´ *** ERROR! Can´´t read paper tape file ***´)
      END
C
C
C *** Find CPM and ESR on next line of TAPE.JIM2.
C *** Calculate DPM using quench correction, then RETURN.
C *** RETURN 1 if EOF, RETURN 2 if error in finding CPM or ESR.
C
      SUBROUTINE CLCDPM(DPM,IFLAG)
      LOGICAL*1 LINE(80)
      INTEGER*2 LENHF
      REAL CPM, DPM, X, Y
      INTEGER CNUMB, DNUMB, ICPM1, ICPM2, IX1, IX2
      COMMON /C1/ CPM, X

C *** If IFLAG = 1, already have CPM and X values
C
      IF (IFLAG .EQ. 1) GOTO 10
C
C *** Read next line in TAPE.JIM2 & scan for ´(´ in A=999999.9( ***
C
1     CALL READ(LINE,LENHF,0,LNUM,0,&999)
      CALL FINDST(LINE,80,´(´,1,1,I,&9999)
      I = I - 8
C
C *** ´(´ shouldn´t be close to the end of the line ***
C
      IF (I .GE. LENHF) GOTO 9999
      CNUMB = 6
C
C *** Convert A=999999.9 to binary (999999 first, then .9) ***
C
      CALL DTB(LINE(I),ICPM1,CNUMB,DNUMB,´=´,&9999)
      I = I + 7
      CNUMB = 1
      CALL DTB(LINE(I),ICPM2,CNUMB,DNUMB,'.',&9999)
C
C *** Add 999999 and .9 to get CPM ***
C
      CPM = ICPM1 + 0.1*ICPM2
C
C *** Back up from end of line to convert S=0.999 similarly ***
C
      I = LENHF - 4
      CNUMB = 1
      CALL DTB(LINE(I),IX1,CNUMB,DNUMB,'=',&9999)
      I = I + 2
      CNUMB = 3
      CALL DTB(LINE(I),IX2,CNUMB,DNUMB,'.',&9999)
C
C *** Add the 0. and the .999 together to get X (the ESR) ***
C
      X = IX1 + 0.001*IX2
10    Y = -1.030*X + 1.040
C
C Calculate DPM
C
40    DPM = CPM / Y
      RETURN
999   RETURN 1
9999  RETURN 2
      END
```